

## End of the line in sight for FDA?

**The new Republican Congress is naturally antipathetic to the US Food and Drug Administration, but radical change will not be easily accomplished, which is just as well.**

THERE are several reasons why the present US Congress distrusts the Food and Drug Administration. One is simple and ideological; the FDA is a bureaucracy, a device for administering government regulations (on the safety and efficacy of new medicines and medical equipment and on the quality of foodstuffs offered for sale). It is staffed by people who, of necessity, add nothing but their salaries and fringe benefits to the gross domestic product. Another reason is that the activities of the FDA most directly affect the pharmaceutical companies chafing at the delays between the development of new drugs and their arrival on the market. When the Congress returns from its summer break, there will be many who will have woken up to the fact that President Bill Clinton's new war against tobacco (see *Nature* **376**, 539–540; 1995) relies crucially on FDA's willingness to classify tobacco as a harmful substance and nicotine as a cause of addiction. The grumbling about FDA in the first year of the new Congress will become louder in the second. Even now, there will be congressmen seeking to win a little publicity for themselves by preparing bills that would abolish the agency altogether.

The reality is that, as always, there is room for reform, but that abolition of FDA would compel the need to reinvent at least substantial parts of it. The contentious issues mostly arise from the procedures for licensing new drugs for sale, by prescription or otherwise. These are the procedures that require manufacturers to spend large sums of money on clinical trials to demonstrate both the safety and the efficacy of the drugs they seek to have licensed. The delays occasioned are in themselves expensive, and may also be held to affect adversely the health of those who might have benefited from them if early licensing had been allowed. Yet the inventor of a new kitchen gadget, or a cleaning fluid, is not required by the present law to demonstrate the safety and efficacy of his products in advance. If they should prove unsafe, those who suffer damage from them can pursue product liability suits in the courts. If they prove ineffectual, the market (aided no doubt by consumer organizations) will quickly recognize that much, whereupon sales will collapse. Why not follow the same principles for medicines?

### Safety

Whatever the logic (and it is not strong), FDA's most severe critics must be aware that it would not carry

weight in the United States. One of the enduring legends of the nineteenth century is that of the snake-oil salesmen appearing regularly at country fairs, milking credulous audiences of their spare cash with promises of relief from this or that ailment. FDA is modern America's answer to those dubious characters. Or is part of an answer; the general craving for good health, longevity and even immortality has provided a rich market for those offering naturally occurring vitamins and natural oils of various kinds as recipes for continued health. The objection to letting modern synthetic medicines find their level in the market is that they do not enjoy the presumption of safety that applies to natural products, and that even the law of tort on product liability does not prevent suppliers from escaping their obligations behind the protection of the bankruptcy laws. In a country that specifies the safety equipment motor-car manufacturers must embody in their products, there is no political possibility that drug manufacturers would be freed from comparable restraints.

### Efficacy

It is relevant, and even now important, that in the early 1960s Senator Estes Kefauver would have gone a substantial step further. In a seemingly endless series of congressional hearings, he set out to devise a way in which manufacturers would be discouraged from seeking licences for "Me-too!" drugs by requiring that the efficacy of a new drug should be demonstrated, in clinical trials, to be superior to others on the market. In the event, that goal would have been impracticable; even the most elaborate clinical trials are rarely so precise. But Kefauver was sustained in his long campaign by a groundswell of public opinion that is unlikely to have vanished in three decades. Even now, the opinion that the effectiveness of newly licensed medicines should be demonstrated in advance is likely to be widely shared by every congressman's electorate.

So is there nothing to be done to simplify the present procedures for licensing new drugs? As Republican luck will have it, the opposite is the truth. Everybody (FDA included) has learned a great deal from the special arrangements made five years ago for the licensing of drugs thought relevant to the treatment of AIDS; when no proven remedies are available, the seriousness of the condition to be treated must moderate the standards to



be satisfied by the proof of efficacy (as distinct from safety) required of a new drug.

Two other considerations arise. First, compounds offered as new medicines whose design is based on a testable physiological rationale deserve an inside track — a partial presumption of efficacy. That is already an important issue in biotechnology, where many of the compounds waiting in the wings for approval are synthetic versions of naturally occurring proteins, but the queue will become more clamant when more is known of the natural function of genes that, in mutated forms, are linked with important genetic diseases. If, for example, further study and understanding should suggest that there is a replacement therapy for Alzheimer's disease, what formal proof of efficacy would be considered necessary?

The second new opportunity is offered by means in which the design of clinical trials can be made less formal. 'Meta-analysis' is the slogan. The truth is that once the safety of a proposed new medicine has been established, an assessment of its effectiveness can be reached by the careful gathering of data in its tentative use. The outcome is neither as clean nor as decisive as that from a regular clinical trial, but none the less useful on that account. A few exercises of this kind might enable FDA (and similar organizations elsewhere) further to speed up the approval of new drugs. The difficulty, for the radical wing in the US Congress, is that neither of these opportunities can be enshrined in new legislation, but must be decided by a technically expert organization — one like FDA, for example. □

## Care in the community

**There will be no quick answer to the British government's call for better care for the psychiatrically ill.**

THE British government's Department of Health has woken up to a scandal that has been simmering away for the past four years — the plain truth that its humane programme for moving people with chronic psychiatric illness from long-stay hospitals into the community at large is not working as intended. Last week, in the wake of two critical reports by differently constituted teams of inspectors, the minister at the department, Mr Gerald Malone, wrote to Britain's hospital trusts urging them to do better. He is right to ask. Whether he or his successors will be satisfied with the response is another matter.

That the policy of community care is enlightened is beyond dispute. In the bad old days, institutionalization was a disease in its own right, but in the absence of palliatives of psychiatric illness, also a necessity of a kind. Now that manic depression and clinical depression are reasonably well dealt with by means of drugs, and even schizophrenia is controlled by the phenothiazines and their derivatives, it makes good sense that those affected should live in circumstances comparable with those that others of their age enjoy. For any government, community care also

offers potentially huge economic benefits; even psychiatric hospitals are expensive to staff and run, while circumstances usually dictate that they should be a public expense. So there is nothing wrong with the ambition that most of those suffering from psychiatric illness should live in the community rather than in hospitals. The difficulties lie in the way in which the policy is carried out.

When the British version of these arrangements was decreed in 1990, it was intended that health authorities and the social services departments of local authorities should devise a joint plan for dealing with the community care of psychiatric patients in their areas. The hope was that there would also be plans for individual patients, offering them programmes of health care, social support and even occupational activity. Last week's report of the Department of Health's own Social Services inspectorate is based on five local-authority social services departments; it says that only one of the five departments had drawn up such a plan, and that even that had been in place for just over a year.

Shameful though that may seem, it is not surprising (nor at odds with anecdotal evidence emerging in the past few years). The Health Service has been in the throes of huge administrative upheaval and in no position to seek out extra work to do, while even those local authorities with expertise in, say, the care of the elderly have not known enough to anticipate the needs of the psychiatrically ill. And among those, it is plain the people with schizophrenia habitually draw the short straw; the Department of Health's Clinical Standards Group (which acts as a kind of auditor of clinical standards) based a detailed survey of the treatment of schizophrenia in eleven health-service administrative units; none was found to be unequivocally "good", although four approached that ranking. Others were frankly "poor" in the quality of the care they provided for schizophrenia.

Extenuating circumstances abound. The administrative changes of the past few years have not helped. The attention paid to the needs of psychiatric patients as distinct from other claimants on public services almost inevitably depends on the fuss that individuals make. And psychiatric illness is still in many places a tainted kind of disability. But the real difficulty is the lack of adequately trained people able and willing to work peripatetically, as community care requires. Sadly, for the psychiatrically ill, that deficiency will not be quickly remedied. When they are found in sufficient numbers, they will turn out to be expensive. Then the government will be wondering whether it can afford the extra cost, and its critics will be saying that it cannot afford not to afford it. That is at least a tangible difficulty. The less tangible, and therefore the less easily remediable, is the persisting suspicion of psychiatric illness even in enlightened administrations such as the British. People with schizophrenia, who are notoriously awkward customers, are especially likely to be short-changed. But wishing that they would go away will serve no purpose. The lifetime suicide rate may be 15 per cent, but numbers are constantly renewed. □

# Lords' report criticizes 'underclass' status of UK contract researchers

**London.** A House of Lords report on research careers for graduate scientists calls on universities to upgrade the pay and status of contract researchers whom it describes as the new scientific "underclass".

The 60-page report (*Academic Research Careers for Graduate Scientists*, HMSO, £12.95), published last week by the House of Lords Select Committee on Science and Technology, warns that the low pay, "gypsy" existence and inferior status of contract researchers — who make up two thirds of research staff in some fields — is undermining the future competitiveness of British science. The absence of a career structure for contract research, it says, once an important staging post on the way to a permanent academic position, is driving the cream of Britain's science graduates into the arms of industry and commerce.

"More than 20,000 contract researchers, most of whom work under conditions inferior to those of established members of academic staff, hardly constitute the best advertisement to attract either the most able science undergraduates into postgraduate work or school leavers to enrol as university students of these subjects," the report says.

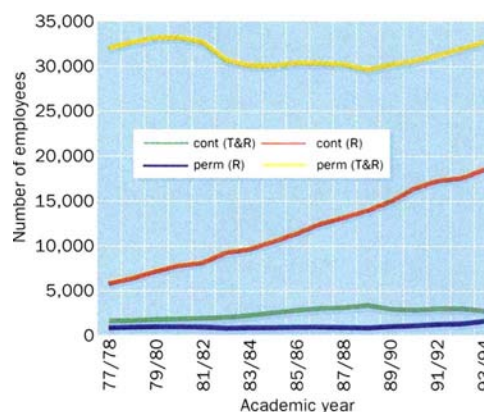
"Universities must get their house in order," according to Lord Dainton, the select committee chairman, who is a former chairman of the University Grants Commission and chancellor the University of Sheffield. "For too long they have been getting staff on the cheap, turning them into gypsies by moving them around on short-term contracts. It is unsettling and an absurdity that will damage science if not rectified."

The report reveals that contract staff now form the bulk of researchers in most science and engineering disciplines. The proportion of contract researchers to total research staff is highest in the fields of biochemistry (64 per cent), pharmacology (61 per cent) and clinical medicine (60 per cent). Metallurgy and materials (58 per cent), pre-clinical studies (56 per cent) and physics (52 per cent) are not far behind. In general women take a proportionately higher share of contracts than men.

Under current practice, research staff recruited on temporary contracts are paid less than their full-time colleagues. According to the Association of University Teachers, "average academic pay is over £27,000 and average research pay is under £18,000".

Contract researchers, similarly, have no pension provision. They are entitled to few of the benefits enjoyed by their full-time peers and have little guarantee of progression to a full-time post. Statistics on contract staff indicate high turnover among the

junior research ranks, but with few filtering through to established positions. Between 1977 and 1994, the numbers of contract researchers at British universities increased by 216 per cent from 5,800 to 18,600. The rise in permanent teaching and research

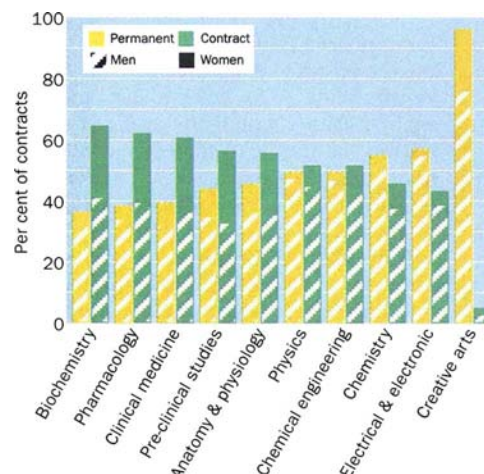


(T&R: teaching and research. R: research only)

**The number of contract researches continues to rise...**

positions for the corresponding period is just 2.3 per cent.

The report recommends that universities should earmark bridging funds to tide over staff who find themselves out of work between contracts. It also encourages universities to break out of the 'six-month syndrome' of appointing staff on a string of short-term appointments. They should, instead, initiate longer-term fellowships in



**... biological sciences suffer most and women suffer more than men.**

line with initiatives already taken by the Wellcome Trust and the University of Warwick.

Interestingly, the report also reproduces evidence from a number of representatives of institutions who pointed out that contract research was not all bad news. The Associa-

tion of Medical Research Charities, whose member charities spent £317 million on research (US\$490 million) in 1993-94, pointed out that short-term research contracts enable "charities to target their support for research to proposals relevant to their objects".

The Royal College of General Practitioners, similarly, said that "a major advantage [of the present system] is that a research worker who goes off the boil or loses enthusiasm or skill can be easily removed without any contractual or redundancy problems".

But in practical terms the report amounts to little more than a set of well-meaning but rather blunt-edged recommendations, which are in danger of being ignored by universities operating in today's climate of too many applicants chasing too few jobs. Signs are already emerging that vacancies for academic staff are likely to rise in the future.

The present bottleneck of staff in the junior ranks is expected to ease within the next decade when a quarter of science and technology academic staff — a legacy of the expansion of higher education during the 1960s — start to retire. Finally, the present expansion in student numbers at British universities is also expected to generate a separate demand for academic staff.

However, the Association of University Teachers warn that many forthcoming vacancies could be left unfilled unless standards for contract researchers are improved. "Some of these researchers will undoubtedly transfer and take up teaching positions," says its spokeswoman, Monica Hicks. But there will be an inevitable shortfall, she says. "People are leaving contract research because there is a limit to the numbers of six-month contracts they are going to tolerate."

The House of Lords report is the second investigation into contract research to be published in the space of a month. The Committee of Vice Chancellors and Principals of UK universities, the Royal Society and the research councils published a joint draft 'concordat' at the end of July aimed at improving the career management and prospects of contract research staff (See *Nature* 376, 289; 1995). This document, which is based on a proposal in the 1993 science white paper, *Realising Our Potential*, has been distributed for comment to universities and research councils. It should be finalized by the end of this year.

**Ehsan Masood**



# Germans divert spent fuel to Dounreay

**London.** A German research institute last week signed an agreement with the Dounreay reprocessing plant in Scotland under which Dounreay will accept fuel elements from its research reactor containing bomb-grade highly enriched uranium (HEU). Under the terms of the Nuclear Non-Proliferation Treaty, these should have been shipped to the United States.

The Hahn Meitner Institute (HMI) in Berlin says it was forced to take the decision because the United States had delayed so long in fulfilling its commitments to take the fuel, and its own storage facilities had become dangerously overloaded.

But the signing of the contract with Dounreay came only one day after a US court of appeals lifted an injunction on the import of HMI's fuel elements.

The Nuclear Control Institute, an independent watchdog group, holds the US Department of Energy (DoE) responsible for forcing Germany to strike a deal with Dounreay, which contravenes international agreements aimed at eliminating civilian trade in bomb-grade uranium.

In 1978, the United States agreed, as part of the 1978 Reduced Enrichment for Research and Test Reactors (RERTR) agreement, to take back spent fuel rods of US origin from foreign research reactors that were prepared to convert from the use of bomb-grade HEU to the less efficient low-enriched uranium (LEU). Most reactors agreed to do this.

But protests from antinuclear pressure groups in the late 1980s caused the United States to renege on its promise to take back the spent fuel. Then, in 1993, the Clinton administration renewed the US commitment to anti-proliferation and called for a full environmental impact statement on the consequences of accepting spent fuel, which is due to be published in December.

In the meantime a backlog of 24,000 spent fuel rods in non-US research reactors built up. Last summer the DoE agreed to accept some of these rods from eight European facilities that had acute storage problems. But the landing of the first shipment of 153 rods was held up when the state of South Carolina argued that the full environmental impact statement should be awaited before any import could be allowed (see *Nature* 373, 547; 1995). After weeks of court battles, the import was finally accepted last September and taken into storage at the South Carolina Savannah River Site.

A remaining injunction on a second shipment of 157 fuel rods, including the shipment of 52 from the HMI, was lifted at the end of June. But because the lifting was subject to the appeal court's final

opinion, which was delivered only last week, the DoE chose not to risk allowing import in case further injunctions left bomb-grade uranium once again unprotected on the high seas.

The delay angered the NCI, which calls it a "highly irresponsible" act by energy



secretary Hazel O'Leary, bearing in mind the urgency of the case. "It put the RERTR in danger", says an NCI spokesman, Alan Kuperman, "and it

makes us worry about the US commitment to take back all 24,000 fuel elements, if we can't muster the courage to import 150."

The HMI says that it could no longer wait for an import licence, which it has still not received, despite statements from the DoE that it is now prepared to authorize shipment, because the court's final judgement came down very strongly in its favour.

"We have always stressed to Washington that we need to get rid of our waste by the end of September", says Bernd Jahn, administrative director of HMI, "so our decision [to go to Dounreay] should not come as a surprise."

But the DoE is apparently still hoping that the HMI will change its mind and send its fuel to Savannah River. This will not happen, says Jahn.

Jahn says that once the immediate pressure on the HMI's storage is relieved, then the institute will consider at leisure all the options for handling waste in the future. This will include discussions with reprocessing plants in Europe. Experience has taught him, he says, not to rely exclusively on a single US option because the strength of local opposition there makes it an unpredictable partner.

The research reactor at the HMI, which provides neutrons for the study of materials, will, however, continue its plans to convert to the use of LEU in the future.

**Alison Abbott**

## Tokyo students organize Internet protest

**Tokyo.** Three graduate students at Tokyo University who set up a home page on the World Wide Web to register protests against France's plans to resume nuclear tests in the Pacific (*Nature* 376, 376; 1995) have had to switch to a commercial Internet site to cope with the deluge of responses.

More than 55,000 people from over 100 countries have recorded their protests on the home page set up on the university computer system last month by Seishi Shimizu and Yuichi Nishihara of the physics department and Kiroh Harada of the engineering faculty.

The home page, which was put on a university server intended primarily for research into elementary particle physics, was getting overloaded with responses, says Nishihara, and this could have limited the number of signatures they could collect.

The three decided instead to move the page to a commercial server run by the company Internet Initiative Japan. "We are paying for the site out of our own pocket money", says Nishihara, "with

money saved by drinking less beer." The students also decided to move the page because they felt it inappropriate to continue using a university server for the protest.

They stopped collecting names on 15 August, the fiftieth anniversary of the end of the Pacific war, by which time the tally had reached 55,769. They are now adding the names of thousands more who signed a chain e-mail letter which two of the students began earlier in the summer but which had to be stopped when their mailboxes became swamped. The final list of protesters will be presented to the French embassy in Tokyo this week.

Nishihara says that they will not continue this activity much longer because they have to work on their MSc theses. But they hope that they will have inspired others to start their own petitions. The page with the text of the protest translated into more than 20 different languages will remain on the server until at least the end of September and can be accessed at <http://www.iiijnet.or.jp/nuke/>.

**David Swinbanks**

# Launch woes ground NASA science spacecraft

**Washington.** Recent problems with two US launch vehicles have created a backlog of scientific spacecraft waiting to reach orbit.

Managers at the National Aeronautics and Space Administration (NASA) say the situation strains their already tight financial reserves and could jeopardize future flight opportunities.

The string of launch delays and failures has also heightened scientists' concern about NASA's plans to fly future spacecraft on unproven vehicles.

The most recent delay is with McDonnell Douglas's Delta 2 rocket, which should have launched NASA's X-Ray Timing Explorer (XTE) astronomy satellite into orbit this week. McDonnell Douglas told NASA a delay was necessary because upgrades were taking longer than expected: earlier this month, another version of the same rocket sent a Korean communications satellite into the wrong orbit — the first time a Delta 2 rocket had failed in fifty attempts.

But Delta's woes are minor compared to those of Orbital Sciences Corporation (OSC)'s smaller Pegasus XL vehicle, which has failed completely on its first two outings. As a result, several NASA satellites, including the Fast Auroral Snapshot Explorer (FAST), the Submillimeter Wave Astronomy Satellite (SWAS) and a Total Ozone Mapping Spectrometer (TOMS), which will measure ozone in the atmosphere, have missed their launch dates. FAST was first due for launch in August 1994 and will next month lose its third launch window in a row.

NASA now has a whole queue of small science missions "in mothballs", according to Daniel Weedman, head of the agency's astrophysics division. "For the time being all we can do is sit and wait, and it's extremely frustrating," he says. Launch delays add significantly to a mission's cost, because science and engineering teams must be kept together for longer than originally planned.

After the second Pegasus failure in June, NASA's administrator, Daniel Goldin, refused to allow his agency's satellites to be launched on the vehicle until it proves itself on at least one successful flight. NASA also asked the launch industry to propose stop-gap alternatives that could help to reduce

the backlog while Pegasus is being fixed. But no other vehicle was available except the Russian-built Cosmos, which was ruled out by political and bureaucratic difficulties with using a non-US launcher.

Laura Ayres, a spokeswoman for OSC, says that the Pegasus investigation team expects to conclude its work in early September and to have the XL flying again by November. An Air Force satellite, REX-2, is likely to be the first payload, but a NASA launch is not expected before next year.

The Delta 2 delay, while not as serious, has already caused some reshuffling of the launch queue for larger Explorer-class missions, and has raised concern that the high-priority Near-Earth Asteroid Rendezvous (NEAR) mission could miss its February launch. The XTE, an X-ray astronomy satellite, has already slipped to October, behind the Canadian-US Radarsat mission which will launch next month. If XTE slips past November it could cause additional

problems, as sensitive detectors on board the craft may have to be removed and refurbished, says Jean Swank, XTE project scientist at NASA's Goddard Space Flight Center.

The combined delays could start to eat into monetary reserves NASA intends to use to develop future Explorer-class missions.

Another worry is that NASA's small science missions — the only size the agency is currently proposing — now find themselves without a reliable ride into space. Another new launcher, the Lockheed-Martin LLV-1, also failed on its first attempt in mid-August. The LLV is due to launch NASA's low-cost Lunar Prospector in 1997.

Several members of the agency's science advisory group have warned of NASA's increasing reliance on an unproved family of mid-size rockets known as 'Med-Lites' to be derived from current OSC and McDonnell Douglas vehicles, and due to make their debut in 1998. NASA, they say, runs the risk of putting too many eggs in the same basket — a situation the space science community knows only too well from its disastrous dependence on the space shuttle over the 1980s, and is learning once again with Pegasus.

**Tony Reichhardt**

## Superphénix consortium seeks compensation

Paris. The three European electricity companies that own the Superphénix fast-breeder reactor at Creys-Malville in France are negotiating with the French government for compensation for loss of income following the conversion of Superphénix last year into a research reactor.

NERSA, the French, German and Italian consortium that owns Superphénix, paid FF27.7 billion (US\$5.5 billion) to build the plant in the 1970s. But the reactor has been plagued by technical problems and operated for a total of less than six months. It was finally shut down in 1990 following repeated leaks in its sodium cooling circuits.

Last year the government approved its conversion into a research reactor, to study mechanisms of plutonium incineration. The FF100 million annual costs of the research programme are being shared by the Atomic Energy Commission (CEA) and the French utility EDF, but three new reactor cores, necessary for the reactor's conversion, will be paid for by NERSA.

The Superphénix reactor was started up again in August last year. But technical problems continued and it was closed down again last December when there were leaks of non-radioactive gas. It was last week given permission to restart by the government at 30 per cent of its maximum output.

Despite the change in the reactor's status, the utilities making up the consortium — EDF (51 per cent), ENEL (Italy 33 per cent) and SBK (Germany, The Netherlands, Belgium and the United Kingdom 16 per cent) — decided earlier this year to extend their participation in NERSA, which hopes that power levels may be progressively increased to original levels, until 2001.

But the utilities are now seeking compensation from the French government because the reactor's main aim is no longer to produce electricity. "It is clear that if the research means that energy output is sacrificed, then the partners need compensation of some kind", says Alexandre Autieri, the deputy-permanent secretary of NERSA, who adds that negotiations are at an "advanced stage".

Successful compensation claims would reduce the financial contributions of the partners, several members of which, including Germany and Italy, are rumoured to be unhappy with the reactor's reduced income. But Autieri says these rumours are false. "To my knowledge, and as far as ENEL is concerned, there is no crisis among the partners", he insists.

**Declan Butler**

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# Japanese plutonium suspected in French tests

**Paris & Tokyo.** Plutonium separated from Japanese spent fuel rods sent to France for reprocessing could be ending up in French nuclear weapons. This is one conclusion of a new report that provides evidence that safeguards by international authorities may not always be implemented. If confirmed, the report would embarrass the Japanese government, which opposes the resumption of French nuclear tests.

The report, commissioned by a group of

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E.P.L./Steve Horgan

The conclusion that the plutonium returned to Japan is not of Japanese origin raises two issues. The first is diplomatic, because Japan opposes the planned French tests in the Pacific. Some Japanese pressure groups last week submitted a petition to the government calling for an end to the reprocessing agreement with France unless firm assurances can be given that Japanese plutonium is being properly safeguarded and not ending up in the French nuclear weapons programme. But the second and perhaps more serious issue concerns the ability of international organizations to verify agreed safeguards concerning the handling of nuclear materials. In principle, spent fuel reprocessed in France is subject to two international safeguard regimes, those of the International Atomic Energy Authority (IAEA) and of Euratom.

But while foreign waste is processed in 103 facilities in France, according to the report, only eight are subject to IAEA control. Moreover, according to an IAEA official cited in the report, the IAEA does not even inspect all eight facilities because of "budget problems", and instead relies for verification on reports from the French authorities. The international agencies have been "publicly misleading people about their technical capacities", says Mycle Schneider, who is one of the report's authors. Schneider claims that nuclear waste sent to France enters a "plutonium pool", where it becomes impossible to trace the ownership of material, and from which it could be removed without detection.

Japan's Science and Technology Agency, which oversees Japan's plutonium policy sees "no problem" with the reprocessing at

La Hague, according to a spokesman. The agency insists that the safeguards of the IAEA are adequate and are being strictly observed at the French plant. Also, they are backed up by Euratom safeguards, it says.

However, the agency does admit that mixing of plutonium from different sources may occur, but says that it is only a requirement that an equivalent quantity of fissile plutonium be returned to Japan as is separated from Japanese fuel.

The IAEA comments that it has no jurisdiction over France, which is a nuclear weapons state, and that it only carries out inspections at La Hague on a voluntary basis. Euratom, which has responsibility for tracing all nuclear materials in the European Union, has greater powers. Its 250 inspectors trace all materials and carry out on-site inspections. But Euratom has no access to military facilities, and a spokesperson admits that the dual civil/military nature of many French nuclear plants complicates the inspection procedures. The possibility that foreign nuclear waste might end up in military programmes cannot be discounted, he says, given the practice of "flag-swapping" equivalent nuclear materials. But he emphasizes that the tracking of amounts of equivalent plutonium is well controlled, and complies with international agreements.

Cogema describes the report's conclusions as an "intellectual construction". Admitting that plutonium from different sources is "obviously mixed" during reprocessing, Cogema asserts that all international agreements and contracts have been respected. It further argues that plutonium handled at La Hague is not weapons grade.

**Declan Butler & David Swinbanks**

## Cogema de La Hague; providing for weapons?

Japanese consumer organizations and pressure groups opposed to nuclear weapons and nuclear power, was produced by the Paris-based World Information Service on Energy (WISE), a consultancy on energy and environmental matters, whose clients have ranged from Greenpeace to the French ministry of environment.

The WISE report argues that because nuclear reprocessing facilities have both civilian and military roles, there can be no guarantee that some plutonium derived from reprocessing of Japanese nuclear fuel has not ended up in French weapons. Japan has agreed contracts worth more than FF20 billion (US\$4 billion) with the French company Cogema for the reprocessing of 2,925 tonnes of spent fuel, which would yield 23 tonnes of plutonium.

Under the terms of international safeguards all of this separated plutonium must be returned to Japan. But the returned plutonium need be only 'equivalent' to that originally sent. Because the isotopic composition of the first large (1.5-tonne) shipment of plutonium returned to Japan in late 1992 does not match the burn-up rate of typical Japanese spent fuel discharged in the 1980s, "it was certainly not of Japanese origin", concludes the report. Cogema's reprocessing plant at La Hague has produced plutonium for the French bomb programme, and has also separated plutonium from Japanese spent fuel. Under Cogema's present system, mixing of military and civilian plutonium could occur.

Moreover, claims the report, there are good reasons to believe that "old" Japanese plutonium derived from fuel reprocessed in the 1970s and 1980s may have been "swapped" for "fresher" material in the 1992 shipment, which is more suitable for MOX (mixed oxide fuel) production.

## Leaked letter confirms UK budget cuts

London. The leaking of an official letter written by the director general of research councils, John Cadogan, confirms that the British science budget will be reduced by £21 million (US\$34 million) in 1996-97 to help pay for the government's technology-related research initiatives.

It suggests earmarking one per cent of this year's budget for next year, for project proposals from research councils that relate to technology-related initiatives such as the Realizing Our Potential Awards Scheme (ROPAS).

"Ministers agree that it would be prudent to have available about £21 million for a further round of the initiatives approved this year as well as for any new initiatives and or realignments which might follow Foresight," Cadogan writes.

John Mulvey from the pressure group Save British Science says the letter's contents are no surprise. "We have long been aware that money for exercises such as

Technology Foresight would have to come from existing budgets that are declining in real terms."

John Battle, spokesman on science for the Labour Party, says the letter was a "betrayal of trust". Labour, says Battle had warned that "the government's commitment to science research has been looking increasingly shaky" following the transfer of the Office of Science and Technology from its Cabinet position into the department of trade and industry.

But Kenneth Pounds, chief executive of the Particle Physics and Astronomy Research Council (PPARC), points out that in May the government pledged £40 million "of new money" over three years for projects linked with Technology Foresight. But he added that research councils were feeling "a little nervous" at the prospect of making room in their budgets for schemes such as ROPAS "without damaging existing research commitments". E. M.

# Crick's resignation heralds new era for Salk

**San Diego.** The surprise resignation of Francis Crick from his brief stay as president of the Salk Institute, announced last week, marks a generational changing of the guard at the research facility in La Jolla, California.

Crick, who is 79, has worked at the institute since 1976 and was president for less than a year. His resignation, coming only weeks after the death of the institute's founder Jonas Salk, ushers in a new era for the institute, which, in recent times, has had difficulties appointing and keeping presidents.

Crick, who shared the Nobel prize with James Watson for their discovery of the molecular structure of DNA, leaves the post this week because of ill-health. He

underwent coronary angioplasty in June. He declines to comment on his resignation, but a press release from the Salk says that he plans to continue his research in theoretical neurobiology at the institute.

Inder Verma, a gene therapy researcher who is vice-chairman of the institute, says that the institute is now casting its net wide for a leading researcher to take over the position. "The currency of the institute is science, so we believe a scientist would be most effective — someone who has vision and a lot of desire to do fund raising", he says. He hopes to see the post filled by November. Given the institute's halting efforts at finding new presidents, this timetable may be a little optimistic.

After Frederic de Hoffmann's 15-year

tenure as president ended in 1988, when ill-health forced his resignation, there was a four-year search for a replacement, with the 81-year-old Nobel prizewinner Renato Dulbecco filling in.

During the hunt, two candidates fell by the wayside, one even rejecting an offer that included a new presidential home to be built adjacent to the institute overlooking the

IMAGE  
UNAMAGABLE  
FOR A CORNABGET  
FOREIGN RIGHT  
REASONS

Salk Institute

## More delays at Mount Graham

**Boston.** An appeals court last month rejected a plea from the University of Arizona and the US Forestry Service to lift an injunction on construction of the Large Binocular Telescope (LBT) at Mount Graham in Arizona. The judgement could delay the beleaguered project for years.

Construction at the site has been blocked since May 1994, following a lawsuit by a coalition of environmental groups who claim that it threatens an endangered species of red squirrel indigenous to the mountain (see *Nature* 370, 407; 1994).

The court ordered a full environmental impact study to be undertaken, something that astronomers fear could result in a series of appeals and counterappeals.

To complicate matters even more, the tribal council of the local San Carlos Apache Tribe, which holds the mountain sacred, but which was officially neutral on the project until very recently, expressed in June its total opposition to the telescope. The Forest Service is now reviewing the tribe's request that the mountain be considered a "Traditional Cultural Property and Sacred Site" subject to protection under the National Historic Preservation Act.

Scientists hardened by the decade-long dispute are taking the latest developments in their stride. Two telescopes are already operating on Mount Graham, and 1.5 acres of spruce fir forest have already been cleared for the LBT. "All that remains to be cleared is one quarter of an acre, on a mountain that has nearly 200,000 acres of National Forest land", says Bruce Walsh, a biologist at the University of Arizona.

Walsh and his colleagues dismiss the red squirrel argument, saying that five years of daily monitoring has failed to turn up any evidence of squirrel activity at the

site in dispute. "It really does seem to be an anti-science battle", says Peter Strittmatter, director of the Steward Observatory.

The LTB consortium, which includes the University of Arizona, the Research Corporation of Tucson and the Arcetri Observatory in Florence, Italy, meets this week to consider its options. One possibility would be to return to the original site on the mountain which had already been approved by the federal government in 1988. The consortium voluntarily abandoned this site because biologists at the University of Arizona suggested that the second site would be less disruptive to the squirrels.

The second possibility is to proceed with the environmental review: "but by the end of it astronomers could be further away from their goal than they are now", says Richard Kvale, District Ranger for the Forest Service.

Work on the telescope is continuing off-site, despite the facility's uncertain future. Final structural designs of the LBT, which are being prepared by two Italian companies, should be completed this autumn. Preparations are also underway to cast the LBT's 8.4-metre glass mirrors at the University of Arizona.

"This telescope is going to be built", insists Strittmatter. "It is too important for it not to be built." Among other things, the LTB will be capable of seeing faint objects in the sky such as early-forming galaxies and sheets of dust around the Sun and other stars.

Greatly needed assistance may come from US Representative Jim Kolbe (Republican, Arizona) who announced last week that he will propose new legislation in an attempt to end construction delays.

Steve Nadis

**Crick: no comment.**

ocean. Brian Henderson, a physician hired largely for his fund-raising capabilities, was appointed but stayed only 20 months.

But there is one major factor that may make the presidential search more productive this time round — the absence of Salk himself. Although he was popular with the general public for his development of the polio vaccine, Salk's relationship with the scientific community was more complex.

After Hoffman left in 1988, Salk assumed a more prominent role at the institute than he had for years. He also helped to found the Immune Response Corporation, a public company that is trying to develop an AIDS vaccine. And Salk became an outspoken proponent of scientific approaches to an AIDS vaccine that were sharply criticized by some leading immunologists.

In his final years, Salk was scorned by some scientists who claimed that he acted as a stock promoter who manipulated research announcements at scientific conferences with an eye more towards the stock market than peer-reviewed publications.

However, at his institute, Salk's presence was "ethereal", says Verma. "It was like going to your parents' house. You sleep well, knowing that they are there. Jonas was like that; his presence was reassuring." But with the patriarch of the house of Salk gone, it might be easier to find a new head of the household; one who won't have to share the seat at the head of the table.

Rex Dalton

# Journal impact factors

SIR — A correspondent earlier this year (*Nature* 374, 492; 1995) asserts that: "... Computerized analyses of scientific publications allow immediate comparison of applicants by objective parameters". Following the recent public competitions for full-time university academic positions (*concorsi*), a group of unsuccessful candidates has levelled accusations at members of the examining commission and demanded that objective approaches, notably the impact factor (IF) and citation index (CI), be adopted in the evaluation of scientific publications of applicants.

The function of the IF is to ascertain the importance of scientific journals and, indirectly, the articles published in them, on the basis of the number of citations they yielded in a period of two years. Applying an IF parameter intended for publications to the university *concorso* context is, in my view, arbitrary. Some of the protesters have quoted global IFs but have not always explained how they are calculated. One procedure is to divide the IF for each article by the number of authors, but it is far from clear whether such a procedure can be correct. Why stop with two years' citations, when figures for the whole of a candidate's career should be available? What has been the candidate's specific contribution to the research described? It is also crucial to assess the relevance of published research to the discipline of the *concorso*: a biologist whose published article in *Nature* obtains a high

article by the number of co-authors.

In view of these objections, the global IF of the dissatisfied applicant drops from 17.76 to 2.97. It is also evident from the graph that the candidate had not carried out any valid scientific activity for the five years preceding the *concorso*. In reality, his publications treat a limited area of the discipline in the *concorso*, which raises some concern about the applicant's knowledge of the whole subject and his ability to teach it.

To entrust the assessment of university *concorso* applicants to a computer rather than a committee is, to my mind, misleading and simplistic. To ignore an applicant's knowledge of and competence in the many subdivisions of his discipline, to overlook his career path, particularly in the few years before the competition, and to dismiss the interest expressed in his monographs and works published in national and European journals where the IF is inadequately assessed is damaging to say the least.

**Giovanni Motta**

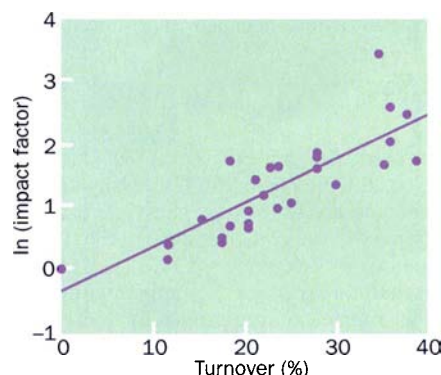
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SIR — Journal impact factors (IFs) are increasingly being used as 'objective' quantitative measures of the quality of output of individuals, research groups or universities, excellence being equated with the ability to publish in journals with high IFs<sup>1</sup>. They are calculated by dividing the number of all citations of articles published in a particular journal during the previous two years by the number of articles published in that journal in those two years<sup>2</sup>.

However, there is a tenfold difference in the IF of the leading journals in different specialist fields, even within the same broad subject. Why is this? I have recently conducted a simple analysis which shows that most of this variation in journal IF is totally independent of the quality of the science. I took a random sample of 28 'good' biological and biomedical journals (defined as those in the top 15% in their specialist field in the IF league tables<sup>2</sup>, excluding general science and review journals). From the first 5 normal research papers in the most recent issue of each I then calculated the mean time from submission to publication (= publication lag; range = 4.4–21.0 months) and the percentage of cited references published within the past two years (=turnover, a measure of how much the discipline relies on recently published material; range = 0–39%). Turnover is similar in concept to

the 'cited half-life' listed (but strangely never used) in IF tables, but is independent of journal quality. IFs were significantly related to both of my parameters (correlation of  $\ln(\text{IF})$  with publication lag:  $r = -0.698$ ,  $P < 0.0001$ ; with angular transformed turnover:  $r = 0.834$ ,  $P < 0.0001$ ; see figure). Both were significant in a stepwise multiple regression predicting IF, indicating that their effects were partially additive (combined  $r^2 = 0.717$ ,  $P < 0.0001$ ). Thus almost 72% of the tenfold variation in IF between leading journals in different specialist areas was explained by two variables that were entirely unrelated to the scientific quality of either the papers examined or the journals!

Turnover and publication lags tend to be broadly similar within, but not between, specialist fields. Given that IFs

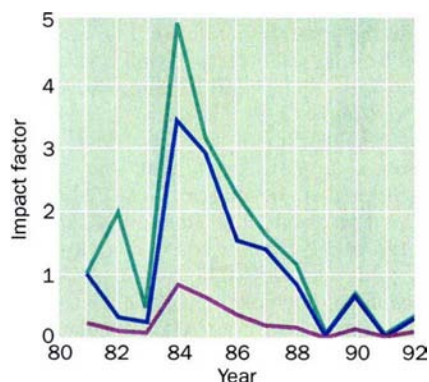


Dependence of journal impact factor (log scale) on turnover of the scientific discipline (angular transformed % of citations within the past 2 years) — see text for details.

are based entirely on citations appearing within two years of a paper being published, the far higher IF of top biochemical and molecular journals compared to (say) ecological ones is therefore largely an artefact, due to (1) publication lags being far shorter (mean of  $6.2 \pm 0.5$  months, c.f.  $16.3 \pm 1.2$ ) and (2) a far greater percentage of their cited references being from the past 2 years (mean of  $28.2 \pm 2.5$  %, c.f.  $9.5 \pm 1.2$  %). While IFs can be used as measures of journal quality within narrowly defined disciplines, they are next to useless in broader-scale comparisons (for example comparing different biological specialisms) unless these systematic biases are corrected. We should ensure that funding bodies and others who use IFs are aware of the unscientific nature of their current measure of scientific excellence

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Green: IF Candidate + co-author (1992 value): sum 17.757. Blue: IF candidate + co-author (annual value): sum 12.555. Purple: IF candidate alone (annual value): sum 2.962.

IF should not as a matter of course win the competition for the chair of urology or otorhinolaryngology.

The graph shows the case of a failed candidate who protested because he claimed his IF was high (about 17,000). That figure was arrived at by obtaining a global value of his career (1981–91), by applying to his articles the IF value that the journals in which they appeared had themselves received in 1992 and by neglecting to divide the IF value of each

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# Plagiarism is worse than mere theft

**A long-delayed report of a meeting on plagiarism in Washington two years ago is none the worse for the delay, but should be a reminder that most institutions are still poorly equipped to deal with it.**

AMONG scholars, plagiarism is the worst of bad behaviour. In the strict sense, when it entails the copying out of other people's words embodying their ideas, it is not just deception but theft — the theft of other people's creativity and of their conception of what the world is like. But plagiarism is also a deception of all who read the stolen texts: not only does the thieving author win credit for notions that are not original, but readers are unable to reconstruct the route by which they have come to see the light of day.

Plagiarism in the literal sense is also the most tangible of academic misdemeanours, which can be dealt with by the simple question, "Have the words been copied, or have they not?" That means that when a plagiarist is unmasked, the case appears open and shut. So it is no wonder that plagiarism is often the most easily punished of academic misdemeanours. People are asked to resign their posts, and often do so meekly. So why does plagiarism remain the most common of accusations of misconduct against academics?

Two years ago, the US Public Health Service's Office of Research Integrity (ORI), the successor of the earlier Office of Scientific Integrity at the National Institutes of Health (NIH), together with various committees of the American Association for the Advancement of Science and the American Bar Association, held a conference in Washington to brood on plagiarism, and it has just circulated an account of the proceedings. Despite the passage of time, the document remains a good read, and a startling one. (ORI says that the text will not be published as ink on paper, but that it will send interested people a disk on request.)

It deserves a wide audience, and not simply for the riveting discussion, by Dr Frederick Newsome of the Columbia University College of Physicians and Surgeons, of the sense in which Pythagoras was a systematic plagiarist. The tale is that Pythagoras had been urged by his elder Thales to travel to Egypt and to attach himself to the priesthood who were then the custodians of the Nile Valley's wisdom. Young Pythagoras did just that, returning to Greece in his early fifties brimful of notions about the immortality of the soul, the square of the hypotenuse of a right triangle and the symbolic significance of simple geometrical shapes that became the hallmark of the Pythagorean School. Newsome tells how upset the

Egyptians were to see their credit stolen.

The contemporary cases of plagiarism raise a different question: how can established academics, almost by definition not neuronally deficient, be so artless in their intellectual burglary? Two of the several cases described in detail at the ORI meeting involve the use by a senior scientist of material from grant applications submitted by others and seen in the course of the senior people's duties as reviewers of grant applications. In both cases, the senior people had taken 'background material' from the grant applications to include applications of their own, even copying over errors in the lists of references, but without attributing them to the authentic source.

One would expect that somebody bent on stealing another's text would at least embellish it with original prose, and that it would be an elementary precaution to make sure that the references are correct. Does guilt, or the wish not to face up to the enormity of the crime being perpetrated, prevent plagiarists from taking elementary precautions against discovery? Certainly they appear as one to hide from the near-certainty that, in the normal course of the peer-review process, their fake will be sent to the plagiarized person for review. That also happened in the well-known case in 1979 when Dr Helen Wachslicht-Rodbard, then at NIH, was sent for review a proposed article from Yale containing six paragraphs (including some crucial mathematics) from an earlier article of her own; she was able to conclude (correctly as it turned out) that the authors of the proposed article were also the authors of a hostile review of her earlier article.

Plagiarists appear also to be amateurish in their attempts to cover up their crime when challenged. One of the scientists who had submitted part of another's grant application as his own claimed that the villain was a postdoctoral fellow once working in his laboratory who had been shown the grant application and who, separately, had been asked to write a document suggesting future directions of research. But the scientist concerned declined to identify the postdoc.

The interest of these cases is not just prurient. They are a reminder that even well-established researchers will do the most outrageous and foolish things in the scramble for repute. The particular value of the ORI discussion is that, in attempting to reach a definition of plagiarism, it knocks on the head several legends behind

which plagiarists are inclined to hide. The idea that unpublished material is fair game for unattributed quotation because it does not enjoy the formal protection of a publisher is both common and false. (The ORI meeting, with all those lawyers in attendance, might have made more of the absolute protection enjoyed by unpublished writings, to which even the 'fair use' provisions of copyright law do not apply.) So is the notion that familiar ideas are in some sense in the public domain so that the language in which they are expressed can be copied freely.

The record of the meeting also clearly brings out the evident difference between scientists' and lawyers' conceptions of what constitutes plagiarism. To lawyers, the crime is akin to copyright violation, to scientists it is more like the commercial crime of 'passing off', by which a manufacturer may seek to claim a distinguished brand-name for inferior goods, or to outright forgery, where an individual may falsify the signature on a large cheque.

So how prevalent is plagiarism? The ORI meeting did not attempt an answer. But if the known plagiarists are so consistently discovered by their amateurish way of working, one is bound to ask how many cases there are of people who have taken more care and escaped detection. Certainly it is a common observation that published articles often refer inappropriately to their antecedents, perhaps crediting an earlier author with a technical development, but not with the insight to which it led him or her. And the steady rumble of complaints from authors originally published in languages other than English that their work has been misappropriated is a sufficiently dense smoke as to suggest that there must be fire somewhere.

What is to be done? Perhaps the chief value of ORI's meeting is that it showed that several universities in the United States have organized themselves effectively to deal with complaints of plagiarism. It will be interesting to see how quickly they can spread themselves to deal with cases other than those centred on flagrant copying. It is more important that other universities should follow suit. And while there are good general reasons for suspecting that making 'research ethics' a formal part of the education of graduate students will serve no purpose, the scale of common self-delusion about plagiarism does seem to require specific instruction of the propriety of correct attribution

**John Maddox**

# Dynamics of stripe formation

Hans Meinhardt

STRIPES are a common feature in developing organisms, but how are pattern elements generated that have a long extension in one dimension and a short extension in the other? On page 765 of this issue, Kondo and Asai<sup>1</sup> investigate the formation of stripes on tropical fish, inviting a fresh look at this process in embryogenesis. Considered in the light of zebrafish genetics, their results open up a

genes is initiated stripe by stripe by specific promoter regions under the influence of particular concentrations of gap gene products (for example, see an early News and Views on the topic entitled "Making stripes inelegantly"<sup>3</sup>).

The important finding of Kondo and Asai<sup>1</sup> is that during growth of a tropical fish, new stripes become intercalated or existing stripes can be split in two. The point of splitting can move in a zipper-like fashion over the field, enlarging the area with an increased stripe number. This dynamic regulation is quite unlike the control of stripe formation by the rigid coordinate system that operates in *Drosophila*. Surprisingly, this stripe-forming mechanism still functions at later stages in many cells.

Some of the regulatory features of stripe formation in fishes have close parallels in the *Drosophila* embryo. The dorsoventral organization is achieved by a high nuclear concentration of the *Dorsal* protein along the ventral side. Thus, the region of high *Dorsal* concentration is a stripe with a long anteroposterior but a small dorsoventral extension. It is brought into position by a repulsive action of the *gurken-torpedo* system, with their high points at the dorsal site<sup>4</sup>. An impairment of the *gurken-torpedo* system can lead to a split of the high *Dorsal* stripe in much the same way as is observed on the growing fishes.

Observations of pair-rule gene activity in the more primitive short-germ band insect *Tribolium* also suggest a dynamic regulation. Rapid cell proliferation takes place close to the posterior pole. New stripes of the proteins encoded by *hairy*<sup>5</sup> or *even-skipped*<sup>6</sup> appear there whenever the distance to an existing stripe becomes too large. In *Drosophila*, with progressing subdivision into more and more cells, a second set of seven *even-skipped* stripes becomes inserted between the primary seven stripes<sup>7</sup>. The new stripes are thinner, as was observed on the fishes.

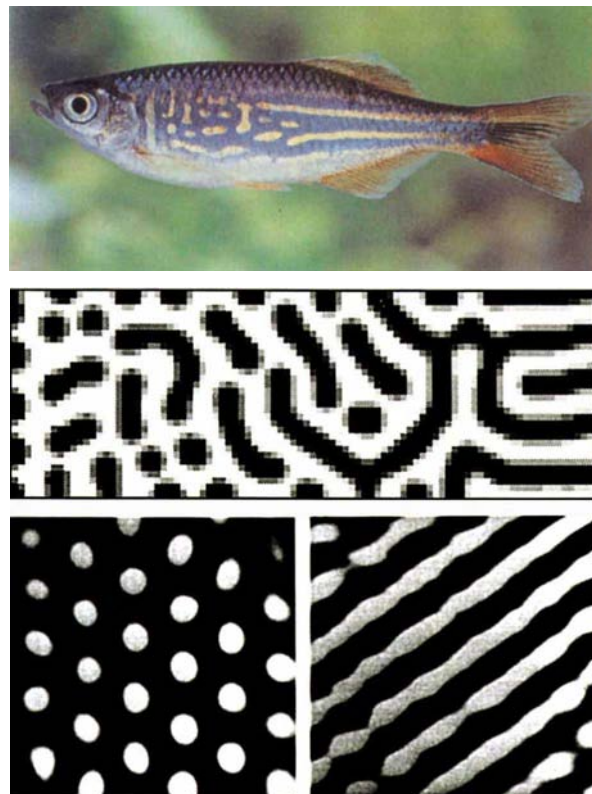
In his pioneering paper<sup>8</sup>, Turing showed that two interacting substances with different diffusion rates can generate

stable patterns in space, the necessary condition for pattern formation being local autocatalysis and a long-range inhibition<sup>9</sup>. Since then, patterns consisting of patches and stripes have been observed in defined chemical systems<sup>10,11</sup>.

What causes stripes instead of patches *in vivo*? Different models<sup>5,12,13</sup> have in common a limitation of autocatalysis at high activator concentrations that is independent of the amount of diffusible antagonist: if the self-enhancing reaction has an upper limit, then the fast-spreading antagonistic reaction must be limited too. Activated cells must therefore tolerate other activated cells in the neighbourhood, leading to an enlargement of the patches until the antagonistic reaction is balanced. On the other hand, an immediate neighbourhood of non-activated cells is essential to maintain the activity of activated cells, either to get rid of the inhibitor or to obtain the necessary substrate.

These requirements of large activated patches and an environment of only non-activated cells seem to contradict each other, but this is not the case: in a stripe-like activation pattern, each activated cell has an activated neighbour and non-activated cells are close by. A variant of this scheme is important for insect segmentation. Stripe formation also occurs if two cell states exclude each other locally but activate each other over a long range. A long common border between two adjacent stripes allows an efficient mutual activation. This prediction<sup>14</sup> has been confirmed for the segment-polarity genes<sup>2</sup>.

It has long been known that local self-enhancement occurs in *even-skipped* and *fushi tarazu*, as expected from the general theory. Other components involved in cell-cell communication are predicted, and two recently discovered pair-rule genes are good candidates for this function<sup>15,16</sup>. So, the rigid mechanism of



Stripes and patches derived from three different systems, neatly illustrating the convergence of results obtained by molecular genetics, computer modelling and chemistry. Stripes and patches in: top, *Danio malabaricus*, a relative of the zebrafish; centre, a computer simulation made with increasing saturation of autocatalysis<sup>13</sup> (going from left to right), enforcing the transition from patches to stripes; bottom, a chemically defined reaction<sup>11</sup>. Photograph of *D. malabaricus* courtesy of Rohan Pethiyagoda.

possible route to a molecular explanation of stripe formation.

Stripes have been a focus of interest ever since the discovery that the pair-rule gene *fushi tarazu* is expressed in seven stripes in the fruitfly *Drosophila*. Stripe-like expression is now known to be associated with most other pair-rule and segment-polarity genes<sup>2</sup> and is also pronounced, in the ocular dominance columns for example, during neuronal development. Various models have been put forward to explain stripe patterning, but these have been undermined by the finding that transcription of pair-rule

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5. Sommer, R. J. & Tautz, D. *Nature* **361**, 448–450 (1993).
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9. Gierer, A. & Meinhardt, H. *Kybernetik* **12**, 30–39 (1972).
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11. Ouyang, Q. & Swinney, H. L. *Nature* **352**, 610–612 (1991).
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16. Colas, J. F., Launay, J. M., Kellermann, O., Rosay, P. & Maroteaux, L. *Proc. natn. Acad. Sci. U.S.A.* **92**, 5441–5445 (1995).

stripe formation in *Drosophila* may be a late evolutionary modification of a genuine patterning process that can be observed in very different developmental situations, including those found in *Drosophila* itself.

But the stripes on the fish still call for more explanation: those shown in Fig. 1 of the paper by Kondo and Asai<sup>1</sup> are very

narrow with respect to the spaces in between. All the models I know of can only produce stripes and interstripes of the same size. □

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## DEVELOPMENTAL BIOLOGY

# The scattered jigsaw

Richard Warn

EVERY so often a new piece of scientific jigsaw fills a gap in the puzzle. One such piece is the paper by Bladt *et al.*<sup>1</sup> on page 768 of this issue, which identifies the *c-met*/scatter factor signalling system as the controller of muscle precursor cell migration in mouse embryonic limb buds.

Scatter factor, also called hepatocyte growth factor (SF/HGF), is a member of the blood serine-protease family<sup>2,3</sup>. It resembles blood plasminogen in both its structure and its mechanism of activation, but lacks any enzyme activity. Its receptor is encoded by *c-met*, a transmembrane protein with a cytoplasmic tyrosine kinase domain<sup>4,5</sup>.

The scatter factor/*c-met* signalling system is now known to have several biological functions. One relates to a previous striking finding that scatter factor acting at *c-met* induces epithelial cell colonies of various types to dissociate or scatter *in vitro* by rupturing cell-cell junctions and enhancing cell motility<sup>6,7</sup>. The scattered cells will move up a concentration gradient of the factor. Scattering is usually induced through a paracrine signalling mechanism: mesenchymal cells such as fibroblasts secrete scatter factor and epithelial cells (such as kidney or lung) expressing *c-met* receptors respond to it.

For any result observed in a Petri dish, the big question is whether there is a corresponding biological phenomenon *in vivo*. To investigate this, Bladt *et al.*<sup>1</sup> created mouse embryos lacking a functional *c-met* gene and therefore the kinase activity, and determined which structures failed to form. In such embryos the future limb-bud myoblasts failed to detach from the precursor somite blocks and never migrated, although the somite muscles themselves differentiated. In normal control embryos the myogenic precursors about to migrate showed strong *c-met* expression. At this time, mesenchymal cells within the limb buds express scatter factor. The conclusion is that scatter factor, secreted from within the limb bud, diffuses locally and induces muscle precursor cells to detach from the somite edges and to migrate up the concentration gradient. A similar failure of migration was found for the muscle pro-

genitors of the diaphragm and the tip of the tongue.

Because scatter factor is also a potent mitogen for many epithelial cell types *in vitro*, it was predictable that some failure of tissue formation apart from muscle would be found. A marked reduction in liver and placenta size is reported by Bladt *et al.*<sup>1</sup>, coupled with a large reduction in the number of liver parenchymal cells and placental trophoblast cells in *c-met*-negative embryos. The results are identical to those described for mouse embryos lacking scatter factor<sup>8,9</sup> and demonstrate that the factor has a major function in forming these tissues.

There is also evidence that the duo of scatter factor and *c-met* is involved in the morphogenesis of many other organs. This has been particularly well documented for the kidney and the mammary gland. As for the mouse limb, the initial findings came from work with cultured cell lines. If MDCK kidney cells are cultured on top of collagen gels and scatter factor is added, cells scatter and invade the gel<sup>10</sup>. But if the same cells are grown inside the gel in the presence of scatter factor, extensive tubulogenesis results, with the appearance of structures in which polarized cells border a lumen<sup>11</sup>. Very similar results were obtained with mammary gland cells<sup>12</sup>. It appears that the cells respond to scatter factor in almost opposite ways, depending on spatial and other differences in the extracellular environment.

There is now good evidence that these effects are not tissue culture artefacts but that they mirror morphogenesis *in vitro*. The addition of scatter factor antibodies to metanephric kidney rudiments grown in serum-free organ culture inhibited the normal branching of the uretic bud rudiment<sup>13</sup>. Scatter factor was expressed *in vivo* by the metanephric mesenchyme and *c-met* was fairly strongly expressed in the cells of the ureteric bud, suggesting a paracrine induction of tubule formation.

Organ culture has also demonstrated that the scatter factor/*c-met* signalling system has a major function in the development of the mammary gland<sup>14</sup>. Furthermore, it acts in sequence with and

in complementary fashion to neuregulin, a member of the EGF family, to induce complete differentiation. Addition of SF/HGF to mouse mammary gland tissue in culture promoted branching of the ducts in much the same way as occurs *in vivo* during its differentiation. It also inhibited secretory protein production. In contrast, neuregulin promotes budding of mammary alveoli and milk protein secretion.

The effects seen in organ culture are paralleled by those *in vivo*. Scatter factor expression occurs throughout mammary gland development in virgin mice and reaches a peak at the time of ductal differentiation; levels then fall during pregnancy and lactation. In contrast, virtually no neuregulin is synthesized in virgin animals but there is a large increase during pregnancy.

In conclusion, the varied effects of scatter factor *in vitro* can now be related to diverse functions *in vivo* during embryogenesis. These effects all seem to be mediated through a paracrine effector system, but there are jigsaw pieces related to possible autocrine functions of scatter factor that do not yet fit to form a picture. Co-transfection of human *c-met* and scatter factor genes into murine 3T3 fibroblasts produces cell lines that become tumorigenic when transplanted into *nude* mice<sup>15</sup>. The transplanted cells show areas of epithelial cell-like differentiation with cell-cell junctions and the presence of tubule lumens. Co-expression of markers for mesenchymal (vimentin) and epithelial (keratin) cell types occurs within the cells. These changes resemble the mesenchymal-to-epithelial transition of cells that occurs during the morphogenesis of a number of organs, including the kidney. Cell lines expressing both *c-met* and scatter factor have been derived from the early stage renal mesenchyme of mouse embryos<sup>13</sup>. But the possible functions of SF/HGF/*c-met* signalling in such autocrine situations remain to be elucidated. □

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# Is the ocean losing nitrate?

Louis A. Codispoti

BECAUSE available fixed nitrogen is a major control on plant growth in the oceans, changes in the oceanic reservoir (which mainly consists of nitrate) may have serious consequences. On page 755 of this issue<sup>1</sup>, Ganeshram *et al.* add to the evidence that the present-day ocean is losing fixed nitrogen. If this is indeed the case, it matters not least because less fixed nitrogen implies lower plant productivity, which in turn implies lower oceanic uptake of carbon dioxide. Together with work by Altabet *et al.*<sup>2</sup> published earlier this year, the new paper may finally convince those who have doubted the view that variations in oceanic denitrification rates can have a considerable effect on concentrations of atmospheric carbon dioxide.

Denitrification is the main sink for oceanic fixed nitrogen. This respiratory process can dominate under suboxic conditions (dissolved oxygen less than 1–2 per cent of saturation), and it converts oxidized forms of nitrogen, again mainly nitrate, to free nitrogen or nitrous oxide which most plants cannot use. The well-developed oxygen minimum zone in the eastern tropical North Pacific Ocean is one of three major identified sites of water-column denitrification, and Ganeshram *et al.* provide evidence for decreased denitrification in this region during glacial periods (we are currently in an interglacial). Their results are analogous to those of Altabet *et al.*<sup>2</sup> who studied the record in the Arabian Sea, another of the principal denitrification zones (the third is in suboxic waters off Peru).

In combination with studies that point to reduced sedimentary denitrification during glaciations<sup>3</sup> and a present-day oceanic fixed-nitrogen budget that may be in deficit<sup>4</sup>, a picture emerges of a system that can oscillate from excess to deficit because of higher interglacial denitrification rates. Changes in the inventory of available fixed nitrogen could affect the ability of oceanic biological processes to sequester atmospheric carbon dioxide by causing increases or decreases in its photosynthetic uptake. A time-varying fixed-nitrogen inventory could, therefore, contribute to the atmospheric carbon dioxide variations observed in ice cores<sup>5</sup>. Higher levels of fixed nitrogen during glaciations would contribute to lower levels of atmospheric carbon dioxide, and vice versa.

This view contrasts with past ideas. Twenty-five years ago studies of the fixed-nitrogen budget in the ocean (estuaries not included) produced 'comfortable' answers. It was usually assumed that the

budget was in a steady state, and the problem was finding a sink for the inputs because the sedimentation rate for nitrogen was too small. Estimates of the denitrification needed to balance the budget averaged about 100 Tg N yr<sup>-1</sup> (1 Tg = 10<sup>12</sup> g), and residence times for fixed nitrogen were set at around 10,000 years.

Because other factors might compensate for changes in denitrification, it is important to determine the state of the present-day fixed-nitrogen budget. If it can be shown to be in deficit by a significant amount, the hypothesis highlighted above (first stated by McElroy<sup>6</sup>) would withstand



The single-cell marine alga *Trichodesmium*. Cells in the anoxic centre of a colony carry out nitrogen fixation.

another test. Anthropogenic activities may have increased inputs to the open ocean (excluding estuaries) by about 50 Tg N yr<sup>-1</sup> (refs 6, 7); but even if we include this anomalous several-hundred-year period in looking at the glacial-interglacial timescale, it is likely to be dwarfed by increasing estimates for marine denitrification. The recent literature<sup>8</sup> and a poll of colleagues suggest that the open-ocean denitrification rate is likely to be revised from about 120 Tg N yr<sup>-1</sup> (ref. 4) to over 250 Tg N yr<sup>-1</sup>. Although other sources and sinks will have modest effects, the issue is likely to boil down to whether or not nitrogen fixation by those few species of marine plants that can use free nitrogen gas is balancing denitrification.

Current estimates of oceanic nitrogen-fixation vary over more than an order of magnitude and are likely to be revised upwards from the 'accepted' value of about 25 Tg N yr<sup>-1</sup> (ref. 6), so the possibility that the present-day oceanic budget is in balance cannot be excluded. Rates high enough to do this would, however, require a drastic rethinking of how the ocean works. Suppose we do find that the budget is currently balanced. Does this mean that after 25 years of effort we will have merely returned to the balanced budgets of the past? I think not, because with the greatly

increased total input and output terms, relatively small negative or positive imbalances could be globally significant. At a minimum, the oceanic fixed-nitrogen budget would seem to be much more dynamic than "dreamt of in our philosophy", with a residence time that has decreased to about 3,000 years. This in turn has implications for the minimum timescales needed to produce significant variation in the budget, and therefore in the timescales over which changes could influence levels of atmospheric carbon dioxide.

The data on the distributions of nitrate and phosphate in the sea indicate a budget that is now in deficit or in a recovery phase from a past deficit. Nitrate is a more frequent limiting nutrient than phosphate<sup>9</sup>, and nitrate/phosphate ratios in most of the ocean are less than the Redfield ratio (16/1 by atoms) of uptake by phytoplankton whereas high ratios are found in localized zones such as the Red Sea<sup>9</sup> (where we know that nitrogen fixation is more important than in most of the ocean). My guess is that once we find techniques to determine denitrification rates in oxygenated water, we will find that they are significant<sup>10</sup> and, once again, will have to revise the overall oceanic rates upwards. Consensus estimates for nitrogen fixation may also rise, but perhaps not as far as some are suggesting.

There is much more to do on all aspects of the budget including investigating the role of trace metals which may control primary production or nitrogen fixation (or both) under some circumstances<sup>11</sup>; in addition, increased denitrification could result if schemes to fertilize the ocean with trace metals led to an increased flux of organic material to depth, as this would increase subsurface respiration and decrease oxygen concentrations. However things pan out, it seems that interesting times lie ahead for those interested in the oceanic fixed-nitrogen budget. □

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# Living with bad architecture

Robin Lovell-Badge

PAPERS in *Nature Genetics*<sup>1</sup> and *Cell*<sup>2</sup>, and another on page 771 of this issue<sup>3</sup>, provide a common link between two very different forms of aberrant growth — benign tumours in humans and the pygmy phenotype in mice. It emerges that both conditions stem from mutations in a gene encoding a so-called architectural transcription factor known as HMGI-C.

In the cell, DNA is packaged into chromatin by histone and non-histone proteins. Chromatin structure varies during the cell cycle and cell differentiation, and with gene expression, and these changes are under the control of architectural transcription factors<sup>4</sup>. Particular attention has centred on the HMG family of proteins<sup>5</sup>, which get their name (high-mobility group; see box) from their small size in gel electrophoresis compared with other non-histone proteins associated with chromatin. They lack activation domains, have little if any sequence specificity in binding, and generally differ from classical transcription factors; instead they are thought to be involved in organizing chromatin at a local level to provide the correct architecture for other transcription factors and the basal transcription machinery to operate<sup>6</sup>.

## Human defects

HMG proteins are in fact very diverse, and HMGI-C forms a subgroup of its own with one other member, HMGI-Y. Both bind DNA sequences rich in adenine and thymine (AT) by domains termed AT hooks, and are fairly ubiquitous during embryonic development but largely absent in adult tissues. Given what has seemed to

be the somewhat general function of HMGI-C, it is surprising that three new studies<sup>1-3</sup> should come up with the protein as the cause of two highly specific but very different growth defects.

Schoenmakers *et al.*<sup>1</sup> and Ashar *et al.*<sup>2</sup> were searching for a gene that could be affected by translocation breakpoints mapping to human chromosome region 12q15. These are often associated with a variety of benign tumours, lipomas in particular. Schoenmakers *et al.* used positional cloning to search within a previously defined 1.7-megabase 'multiple aberration region' (MAR), which was thought to contain the tumour-associated gene, and found *HMGI-C*. Ashar *et al.* had already considered *HMGI-C* as a reasonable candidate for the tumour-associated gene: they knew that it mapped to the MAR; it is required for retrovirally induced neoplastic transformation<sup>7</sup>; and transcription factors have been identified at breakpoints in a variety of tumours<sup>8</sup>.

Both groups determined the gene's organization. The first three exons each encode an AT-hook-motif DNA-binding domain, and the last two encode an acidic region. Importantly, there is a large intron of over 25 kilobases in length between exons 3 and 4 within which most translocation breakpoints had occurred.

More cases need to be analysed, but the common theme emerging is that the cause of the lipomas and other benign tumours is the generation of fusion proteins in which the AT-hook DNA-binding domains of HMGI-C are linked to a regulatory domain from another gene in the translocation partner chromosome. In one case

the latter was a highly acidic, serine-and-threonine-rich domain from a new gene, which could be turning HMGI-C into a transcriptional activator. In a second lipoma studied by both groups, the RNA was found to stem from the first three exons of HMGI-C and two tandemly arrayed LIM domains; these are involved in protein-protein interactions, and could recruit other transcriptional activators to the DNA sites bound by the AT hooks of HMGI-C. The benign nature of the tumours needs an explanation, but HMGI-C is expressed at very low levels. Also, why do they tend to be lipomas? Perhaps HMGI-C is particularly important in adipose tissue, which brings us to the second part of the story.

## Mouse defects

Zhou, Chada and co-workers<sup>3</sup> were interested in the cause of the *pygmy* mutation in mice. Homozygotes for *pygmy* have adult body weights that are 40 per cent, and heterozygotes 80 per cent, of those of their wild-type littermates. Chada and colleagues had previously identified a new allele at *pygmy* by transgenic insertion<sup>9</sup>. In the latest study<sup>3</sup>, a flanking marker from the insertion site was cloned and used to initiate chromosome walks. The authors identified a common region of fifty-six kilobases that was deleted in the mutant alleles, and exon amplification was used to identify a gene within the deletion — the gene turned out to be *Hmgi-c*. Because other genes could have been affected by the deletions, the authors used gene targeting by homologous recombination to create a much more specific null mutation in the gene, thus confirming that the *pygmy* phenotype results from lack of HMGI-C.

How is this phenotype explained? Analysis of *Hmgi-c* expression showed

## The HMG family of proteins

THE HMG proteins are a collection of small acidic proteins that were first described many years ago with the advent of gel electrophoresis techniques for separating macromolecules. Their name, which stands for high-mobility group (and not Her Majesty's Government) proteins, simply reflects their small size (all have relative molecular masses under 30,000) compared with other non-histone chromatin-associated proteins — a terminology that conceals the diverse structure and function of these proteins.

One class is represented by proteins such as HMG-1, which bind bent DNA structures irrespective of sequence<sup>15</sup>. This binding occurs through several domains, first identified by comparison of HMG-1 with UBF, a protein involved in regulating the expression of genes encod-

ing ribosomal RNA<sup>16</sup>. These domains were termed HMG boxes, which unfortunately is rather confusing as not all HMG proteins have them and because similar domains are found in many other proteins that fall outside the original definition. Many of these, including factors important for decisions of cell fate in development, such as LEF-1, TCF-1, SRY and the related SOX proteins, have single HMG-box domains, with the additional property of sequence-specific binding to linear DNA.

On binding, these proteins cause DNA to bend through large angles, which may facilitate the action of transcription factors bound nearby<sup>6,17</sup>. For this reason they can be considered as architectural proteins, although some also have strong activation domains and may contribute

directly to the activation of target genes<sup>18</sup>.

A second class of HMG proteins is represented by HMG-14 and HMG-17 (refs 4-6). Like HMG-1, these do not appear to activate or repress transcription of other genes directly, but they have different DNA-binding domains (random-coil secondary structure) and show affinity for DNA within nucleosomes of active chromatin. HMGI-C and HMGI-Y represent a third class and use domains termed AT hooks to bind A+T-rich DNA sequences associated with many genes<sup>4-6</sup>. Again, they are thought not to play a direct role in transcriptional activation, but participate in organizing complexes of other transcription factors, which in turn recruit the basal transcriptional machinery.

R. L.-B.



that transcripts are present at low levels from birth but at high levels during embryonic development in all tissues except brain. Pygmy mice show approximately normal brain sizes, which is consistent with this finding. Lack of HMGI-C therefore seems to result in a general, cell-autonomous reduction in growth and, in line with this, cultured fibroblasts from pygmy embryos divided much more slowly than did wild-type fibroblasts<sup>3</sup>. A link with cell proliferation was already evident as the DNA-binding ability of HMGI-C can be regulated by phosphorylation through the cell-cycle-dependent p34<sup>cdc2</sup> kinase<sup>10</sup>. Although *Hmgi-c* seems to be required for all cell types except those in the brain, it may be especially important for adipose tissue. Pygmy mice show a disproportionately reduced content of body fat — only about 12 per cent of that of wild-type littermates. Perhaps this is why lipomas are the most frequent tumour associated with the 'neomorphic' mutations of *HMGI-C* described above.

These findings can be taken two ways. Some may consider it a surprise that lack of an abundant, embryonically expressed protein, which is thought to participate in the transcriptional regulation of many genes, results in a relatively mild phenotype (the explanation could be that HMGI-C function is compensated for in part by HMGI-Y). But I think that it provides an ideal example of architectural proteins as facilitators. In this case, HMGI-C provides a link between gene expression and the cell cycle, loss of function resulting in a general slowing down of cell replication and gain of function resulting in a slight increase in division and benign tumours. So regulation of *Hmgi-c* expression, or related factors, could explain much of the difference in growth rate between embryos and adults, between different tissues and between species.

Where to next? Clearly the role of HMGI-C in cell proliferation needs to be

better understood. It would be useful as it might allow clonal analysis in mice in a way that is similar to the use of *minute* mutations in *Drosophila*<sup>11</sup>. This would involve restoring *Hmgi-c* expression in cells of pygmy embryos while creating a mutation in the gene under study.

There is also the possibility that mutations in other genes encoding architectural proteins are associated with specific human diseases. Schoenmakers *et al.* point out that *HMGI-Y* maps to human chromosome 6p21 and that this region is associated with frequent translocations

seen in other solid tumours and lipomas. Moreover, there are many proteins that act as higher-order architectural factors which should be considered; for example, the products of *polycomb* and *brahma*-like genes have a central role in both *Drosophila* and mammalian development<sup>12-14</sup>. In all, we should remember how important architecture is to the quality of life. □

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## EVOLUTIONARY BIOLOGY

# Speciation in monkeyflowers

Jerry A. Coyne

In this era of the genome project and the hunt for cancer genes, genetic mapping might seem to be the bailiwick of Big Science. Beginning with the work of Dobzhansky<sup>1</sup>, however, this approach has long helped evolutionary biology as well as the study of the genetic basis of adaptation and speciation. Up to now, Dobzhansky and his heirs have been hampered by two problems: a paucity of genetic markers, resulting in chromosome maps of low resolution, and the availability of only a few 'model' species, such as *Drosophila*, which do not necessarily possess the traits that excite evolutionists. Both problems have now been eliminated by the advent of molecular technologies that yield large numbers of variable markers and can be applied to species previously refractory to genetic analysis<sup>2</sup>. A study of two species of the monkey-flower *Mimulus*, reported by Bradshaw *et al.* on page 762 of this issue<sup>3</sup>, clearly shows the value of this approach. It represents the first exhaustive use of DNA polymorphism to answer a long-standing question: how much genetic change is needed for the evolution of a new species? The answer is, in this case, surprisingly little.

To most evolutionists, the origin of species is equivalent to the origin of those factors — such as mate discrimination and hybrid sterility — that prevent hybridization of different groups living in the same area. Bradshaw and his colleagues<sup>3</sup> identified two North American species of *Mimulus* apparently isolated only by a difference in pollinators. Although perfectly interfertile when crossed in the

greenhouse, these species are not known to hybridize where they co-occur in nature. Cross-pollination is prevented by the behavioural fidelity of pollinators and by large differences in floral morphology. The hummingbird-pollinated *M. cardinalis* has bright red flowers, large quantities of dilute nectar, a narrow, beak-shaped corolla tube, and protruding anthers and stigmas that place and gather the pollen from the bird's forehead (see photo).



A rufous hummingbird (*Selaphorus rufus*) dining on nectar from *Mimulus cardinalis*. The flower has a deep, tubular corolla that can be probed by hummingbirds but excludes bumblebees. Pollen is placed on and received from the bird's forehead by the exerted anthers and stigma. The red-orange colour is attractive to hummingbirds but cannot be seen by bees.

Flowers of the bee-pollinated *M. lewisii*, on the other hand, are pink with yellow nectar guides, landing platforms for the bees, low quantities of concentrated nectar, and enclosed anthers and stigmas. Because the reproductive isolation between these species is a by-product of their adaptation to pollinators, a genetic study of the floral characters is at once an analysis of speciation and adaptation.

To mark the chromosomes for genetic analysis, the authors used 153 randomly amplified polymorphic DNA (RAPD)

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markers that differed between the two species. Selfing the  $F_1$  hybrids, they produced a large  $F_2$  progeny in which linkage relationships among RAPDs were mapped to the species' eight chromosomes. By scoring these same progeny for eight floral and nectar characters, the authors determined which chromosome regions are responsible for the morphological divergence. Such molecular mapping has been used to analyse differences between crop plants and their wild ancestors<sup>4,5</sup>, but these differences, reflecting artificial selection, say little about evolution in the wild.

The new *Mimulus* results are striking: every one of the eight character differences involved at least one gene of major effect (defined by the authors as a small region explaining at least a quarter of the phenotypic variance among  $F_2$  plants). Moreover, one character difference (the carotenoid pigment affecting flower colour) mapped to only a single chromosome region; and for two others (nectar volume and corolla width), more than half the variance was explained by only one region. Bradshaw *et al.* conclude that the differences in floral morphology isolating these species are based on the evolution of relatively few genes. (It is possible, but not likely, that the large effects of some chromosome segments actually represent several closely linked genes of smaller effect.)

Such studies will eventually resolve one of our oldest controversies, whether adaptations are almost always based on a very large number of mutations, each of small effect (the 'polygeny' view adumbrated by Darwin and later championed by Ronald Fisher<sup>6</sup>), or occasionally on single genes of large effect (this is not, to be sure, the full-blown saltationism of Goldschmidt, but a more temperate view that single genes may produce major portions of adaptations<sup>7</sup>). There is increasing evidence for the importance of major genes<sup>8</sup>, but there are still only a handful of relevant studies, of which the best is now the work on *Mimulus*.

One might be tempted to conclude, as do the authors, that because only a few genes are involved, the speciation event might have been quite rapid. There is, however, no necessary relation between the mode of inheritance and the rate of evolution, for other factors, such as population size and the strength of selection, are crucial.

The authors intend to measure selection on floral characters by the unique experiment of taking potted  $F_2$  progeny into their natural habitat, observing how they are pollinated, and measuring the attendant effects on plant reproduction. It may then be possible to estimate the fitnesses attached to each mutation. This experiment will also test the authors' scenario for evolutionary change: an initial, bee-

pollinated ancestor undergoing three successive steps affecting flower colour, nectar volume and pistil length. This scenario is somewhat problematic, as the first mutant individual with a new colour would seem to be at a disadvantage. (It would attract hummingbirds, but the flower's reproductive organs would foster cross-pollination with the ancestor. There would also be no other red individuals to receive pollen.) Moreover, it would be difficult to select for two of the derived traits because they are based on recessive alleles<sup>9</sup>. These problems might, however, be alleviated by the small amount of selfing known to occur in nature, which not only facilitates the fixation of advantageous recessive alleles<sup>10</sup>, but also allows a single mutant individual to produce others.

The advent of DNA markers means that genetic analysis of adaptation and speciation can be extended to any species that can be crossed to produce some fertile

hybrids. Plants will be particularly attractive subjects for this work, because many groups, such as orchids, are replete with crossable species adapted to different pollinators. Such studies will at last allow us to assess Darwin's famous dictum of gradualism, "*Natura non facit saltum*". □

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## SIGNAL TRANSDUCTION

# Team blue sees red

Henry R. Bourne

UNTIL recently, the molecular switches responsible for cell signalling were neatly divided into a few discrete clans, each comprising a distinct set of protein families that pays attention to a particular set of extracellular stimuli and mediates distinct cell responses. One such clan (blue in the figure) uses intracellular targets of trimeric G proteins and  $Ca^{2+}$  to transduce signals from neurotransmitters and hormones into contractile and secretory responses. Another (green) uses tyrosine kinases, adaptor proteins and Ras protein to transduce signals from growth factors into regulation of cell proliferation and differentiation.

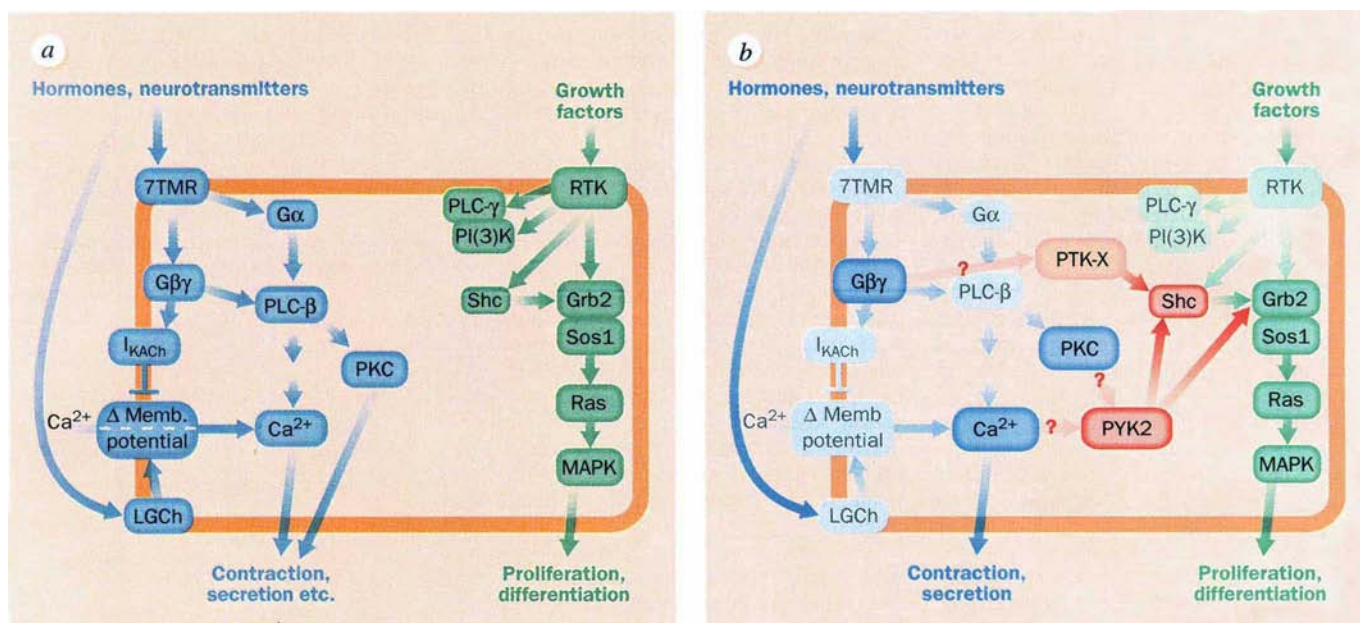
We used to imagine that each clan kept pretty much to itself (part *a* in the figure). From the beginning, however, and right under our noses, blue and green clan members have been actively engaged in physiological transactions. To previous reports on this commerce<sup>1–6</sup> we can now add papers by Lev *et al.*<sup>7</sup> and van Biesen *et al.*<sup>8</sup> (pages 737 and 781 of this issue). The new work implicates Shc, an adaptor protein in the tyrosine kinase/Ras clan, as a key go-between that relays messages from the blue to the green clan (part *b* in the figure).

Signals transmitted by the blue clan come from neurotransmitters and hormones that stimulate either of two classes of receptors — G-protein-coupled receptors, composed of seven-helix transmembrane regions, or ligand-gated ion channels. Downstream signals from both kinds of receptor often converge on con-

trolling the concentration of cytoplasmic  $Ca^{2+}$ , which triggers contraction and secretion in many cell types. Each seven-helix transmembrane receptor activates a specific class of trimeric G proteins, including  $G_q$ ,  $G_i$  or many others.  $G_{\alpha_q}$  and/or  $G_{\beta\gamma}$  subunits activate phospholipase  $C\beta$ , resulting in activation of protein kinase C and an increase in the levels of cytoplasmic calcium by release of calcium from intracellular stores.

Neurotransmitters such as glutamate or acetylcholine elevate cytoplasmic  $Ca^{2+}$  indirectly, by binding to ligand-gated ion channels and increasing influx of extracellular  $Na^+$ . Resulting depolarization of the plasma membrane triggers influx of extracellular  $Ca^{2+}$  through voltage-gated  $Ca^{2+}$  channels. Activation of  $G_i$  proteins by seven-helix transmembrane receptors antagonizes this effect indirectly, by activating  $K^+$  channels such as the inwardly rectifying acetylcholine-regulated channel,  $I_{K_{ACh}}$ , which is found in neurons and cardiac pacemaker cells; the resulting hyperpolarization of the membrane acts to inhibit opening of voltage-gated  $Ca^{2+}$  channels.

To mediate different extracellular stimuli — growth factors, for instance, and an increasing number of intercellular regulators of embryonic development — switches and pathways in the green signal clan converge on regulation of cell growth and differentiation. Many of these pathways depend upon phosphorylation of tyrosine residues in receptor tyrosine kinases, downstream enzymes (phospho-



Shc, the go-between. *a*, The signalling proteins (blue) that control contraction and secretion have long been thought of as belonging to a rather exclusive clan that communicates rarely or not at all with the green clan, which regulates cell growth and differentiation through tyrosine kinases and Ras. *b*, Recently discovered (red) and postulated proteins (pink) pass regulatory messages from the blue to the green

clan, harnessing ligand-gated ion channels (LGChs), receptors with seven-helix transmembrane regions (7TMRs), and G proteins to regulation of the Ras pathway. *I*<sub>KACH</sub>, inwardly rectifying acetylcholine-regulated channel; PLC, phospholipase C; PKC, protein kinase C; RTK, receptor tyrosine kinases; PI(3)K, phosphatidylinositol-3-OH kinase; MAPK, mitogen-activated protein kinases.

lipase C $\gamma$  and phosphatidylinositol-3-OH kinase), and/or adaptor proteins such as Shc and Grb2. A pivotal downstream element in the green clan is the Ras protein, a ubiquitous GTPase that stimulates cascades of other GTPases and protein kinases, including a kinase cascade terminating in activation of mitogen-activated protein kinase. In many cells Ras is turned on by Sos1, a guanine-nucleotide-exchange protein.

Lev *et al.*<sup>7</sup> report that a newly discovered protein tyrosine kinase, PYK2 (red in the figure), acts as a switch that relays messages from protein kinase C and cytoplasmic Ca<sup>2+</sup> to Shc, thus activating Ras-mediated regulation. Although Lev *et al.* cloned PYK2 by a route unrelated to its physiological significance, the almost exclusive expression of its messenger RNA in the brain provided an important clue, leading these investigators to test the potential signalling role of PYK2 in PC12 cells, a neural cell line.

They find that four kinds of stimuli markedly increase phosphorylation of tyrosine residues on PYK2. Activation of a ligand-gated ion channel, the nicotinic acetylcholine receptor, and depolarization of the membrane by increased extracellular K<sup>+</sup> produce this effect; both stimuli require influx of extracellular Ca<sup>2+</sup>. Bradykinin, a peptide that acts on a G<sub>q</sub>-coupled seven-helix transmembrane receptor, also induces increased tyrosine phosphorylation of PYK2, presumably through phospholipase C $\beta$  and consequent transfer of Ca<sup>2+</sup> from intracellular stores to the cytoplasm; the bradykinin

effect on PYK2 does not require extracellular Ca<sup>2+</sup>. Finally, a drug that stimulates protein kinase C exerts the same effect on PYK2 phosphorylation.

Lev *et al.* present good evidence that Ca<sup>2+</sup>-activated PYK2 stimulates the cascade of mitogen-activated protein kinases by phosphorylating Shc and thereby recruiting the Grb2/Sos1 complex: stimuli that increase PYK2 tyrosine phosphorylation also increase tyrosine phosphorylation of Shc, binding of phosphorylated Shc and phosphorylated PYK2 to the Grb2/Sos1 complex, and activation of the kinases. A PYK2 mutant that cannot catalyse tyrosine phosphorylation produces none of these effects, and overexpression of this mutant prevents activation of mitogen-activated protein kinases in response to stimulation of the nicotinic acetylcholine receptor, presumably by preventing endogenous PYK2 (or a PYK2-like protein) from associating with rate-limiting proteins in the pathway. The tale remains nonetheless incomplete, in that Lev *et al.* have not identified the protein or proteins responsible for mediating Ca<sup>2+</sup>-dependent PYK2 activation, and for recruiting the two key characters, PYK2 and Shc, to the plasma membrane (to which they must move in order to bring Grb2/Sos1 together with Ras).

Although several characters are different, van Biesen *et al.*<sup>8</sup> tell a remarkably similar story, connecting G $\beta\gamma$  to tyrosine phosphorylation of Shc and activation of Ras through Grb2/Sos1. A connection had to exist, because many laboratories had previously shown that stimulation of

seven-helix transmembrane receptors coupled to G<sub>i</sub> proteins can promote Ras-dependent activation of the mitogen-activated protein kinase pathway and cell proliferation. In several cell types, G<sub>i</sub>-mediated activation of that pathway is mediated by the G $\beta\gamma$  subunit, rather than by G $\alpha$ , because Ras activation can be prevented by expression of a receptor kinase fragment, known as  $\beta$ 1ct, which binds to and sequesters free G $\beta\gamma$  (for references, see van Biesen *et al.*<sup>8</sup>).

In transient transfection experiments in COS-7 cells, van Biesen *et al.* now show that G $\beta\gamma$  promotes Ras activation by way of Shc and the Grb2/Sos1 complex. Stimulation of two G<sub>i</sub>-coupled receptors, as well as simple co-expression of excess G $\beta$  and G $\gamma$  subunits, induce tyrosine phosphorylation on Shc, association of Shc with Grb2/Sos1, and an increase in the guanine-nucleotide-exchange activity (presumably due to Sos1) associated with Shc; all of these effects are blocked by  $\beta$ 1ct; in addition, the receptor-mediated responses are blocked by pertussis toxin, which inhibits interactions of G<sub>i</sub> with seven-helix transmembrane receptors. The same effects are also blocked by expression of a dominant-negative Sos1 mutant specifically designed to prevent cellular Sos1 from interacting with Grb2, and inhibition by the mutant can be overcome by expressing excess wild-type Sos1. The latter results rule out the obvious (but incorrect) hypothesis that G $\beta\gamma$  activates Sos1 by recruiting it directly to the plasma membrane, without the aid of Grb2.

The data of van Biesen *et al.* provide no



clue as to the molecular pathway by which  $G\beta\gamma$  stimulates Shc phosphorylation. The unidentified tyrosine kinase — I call it PTK-X (part *b* in the figure) — responsible for phosphorylating Shc could turn out, like its functional cognate in the  $Ca^{2+}$ -to-Shc pathway, PYK2, to belong to the Fak subfamily of tyrosine kinases. Perhaps it will emerge that membrane-bound  $G\beta\gamma$  binds to PTK-X and stimulates it directly, an arrangement that would provide the necessary bonus of recruiting Shc (and therefore Grb2/Sos1) to the plasma membrane.

Once PTK-X is identified, will it be found to co-exist with PYK2 in PC12 cells and brain neurons? If so, the two kinases are unlikely to be stimulated at the same time in a single cell, even though both use Shc to transmit 'blue' messages to the 'green' pathway. This is because the postulated kinase and the recently cloned kinase respond to stimuli that act to antagonize one another within the blue clan.  $G\beta\gamma$  released from activated  $G_i$  not only turns on the mitogen-activated protein kinase pathway via PTK-X but also stimulates  $I_{K_{ACh}}$  (refs 9, 10), causing the membrane to hyperpolarize; this effect directly opposes membrane depolarization triggered by the stimulators of ligand-gated ion channels that activate PYK2.

Another observation by Lev *et al.*<sup>7</sup> suggests that — as well as mediating a connection to the green pathway —  $Ca^{2+}$ -mediated activation of PYK-2 would act to potentiate effects of ligand-gated ion channels in a sort of positive feedback loop, within the blue clan: PYK2 inhibits  $K^+$  permeability by phosphorylating tyrosines on a  $K^+$  channel distinct from  $I_{K_{ACh}}$ ; as well as reinforcing depolarization responses to ligand-gated ion channels, this inhibition could at the same time antagonize  $G\beta\gamma$ -mediated hyperpolarization, through  $I_{K_{ACh}}$ , which is stimulated by seven-helix transmembrane receptors coupled to  $G_i$ .

Wheels within wheels! And just what we are coming to expect of the bustling communication networks within and between clans of signalling proteins in the average cell. □

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## Flux lattice makes a hesitant breakaway

P. H. Kes

WHEN a viscoelastic medium is forced to move against random pinning forces, the two extreme stages of the process are clear. When our attempts are weak, the pinning forces will win and the medium will only respond elastically. When we push hard enough, the medium will flow uniformly and experience hardly any pinning forces. The intriguing questions remain for the stage in between, when we want to know in detail what happens at the first breakaway. The report by Yaron and collaborators<sup>1</sup> on page 753 of this issue shows that, in the special case of flux pinning in a type II superconductor, the breakaway is actually a two-step process. The uniform flow is preceded by a very slow, thermally activated plastic motion of the lattice. In other words, the medium hesitantly sets out to move, not by a unanimous breakaway, but by local little jumps.

The 'elastic medium' studied by Yaron and collaborators is a lattice of flux lines in a single crystal of  $NbSe_2$ , a material which becomes superconducting at temperatures below 7.4 K.  $NbSe_2$  is a type II superconductor: below the critical temperature, when an external magnetic field is applied, the field penetrates the material in quantized bundles of flux known as magnetic vortices or magnetic flux lines (see Fig. 1). Any applied current induces a force on these lines. For practical applications, the flux lines must be pinned; if they are free to move in response to the current, they will dissipate energy, destroying the superconducting state. Hence the interest in how, and at what critical current, the pinning starts to break down.

$NbSe_2$  provides a convenient weak-pinning environment, and expert crystal growers in the team were able to grow large single crystals. The latter were needed for sufficient resolution to probe the structural correlations of the vortex lattice by small-angle neutron scattering (SANS) at the neutron facility in Risø, Denmark. With SANS, the group was able to look inside the material and measure the evolution of disorder in the flux-line lattice as a function of the driving force exerted by the transport current that they applied to the crystal. In addition, the measured voltage told them the average velocity of the vortex lattice.

In principle, the amount of structural disorder follows from the widths of the neutron diffraction peaks (Fig. 2) and the rocking curves (the curve of diffracted intensity against angle as the lattice of flux lines is rocked through the Bragg angle).

But it turns out that in  $NbSe_2$  the pinning is so weak that the instrumental resolution only allows the disorder to be resolved in the direction of the flux lines. The correlation length that Yaron *et al.* obtain from the width of the rocking curve tells us how far one can move along a flux line before losing track of its original position. The typical length they find is 11  $\mu m$ , which is about 100 times the separation of the flux lines.

Their main results, summarized in Figs

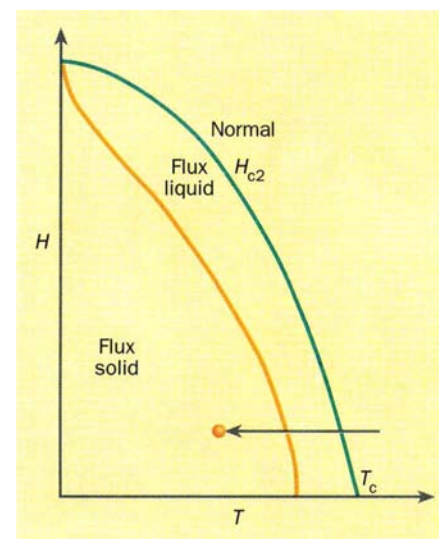


FIG. 1 Phase diagram for type II superconductors such as  $NbSe_2$ . The green  $H_{c2}$  line shows the magnetic field  $H$  which separates the normal state from a state containing a disordered array of magnetic flux lines known as a flux liquid. The orange line (melting line) marks the transition to the flux-line 'solid', in which the flux lines are fairly highly correlated but are disordered by the presence of pinning centres. The arrow shows the field-cooling procedure to the point at which Yaron and co-workers made their measurements, well below the melting line for the flux lattice.

2 and 3 on pages 754 and 755 of this issue, are that the elastic response of the vortex lattice at small currents (driving forces) does not yield a detectable voltage, although it is measured with a resolution far down into the picovolt range. The longitudinal correlation length does not change at this stage either. When the current is high enough for the first vortex motion to be detected, the correlation length starts to decrease by about 20 per cent, revealing a slight disordering of the vortex lattice which is interpreted as an indication of thermally activated plastic flow. Once uniform flow sets in, this turns into a strong recovery of the correlation



length and a recrystallization of the moving lattice.

A remarkable feature of the experiment is that the lattice disorder does not reappear upon decreasing the driving force (the applied current) back to zero. This shows that the low-temperature, dynamic annealing procedure yields a vortex configuration with lower free energy than the original field-cooling procedure used to prepare the specimens. To understand

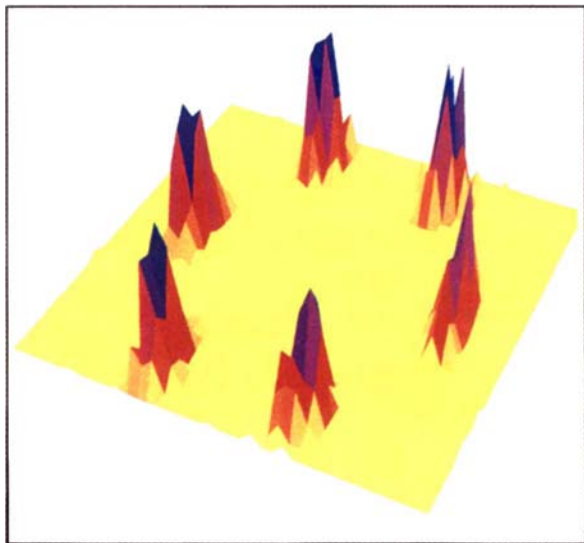


FIG. 2 Scattered neutron intensity on the plane of the detector for a small-angle neutron scattering experiment<sup>1</sup> in NbSe<sub>2</sub> at 1.5 kOe and 4.8 K. The peaks are the six first-order Bragg scattering reflections due to the interaction between the hexagonal magnetic flux-line lattice in the sample and the neutron spin — an effect first predicted by de Gennes and Matricon in 1964. (Courtesy of U. Yaron.)

this, one has to look at the phase diagram of a type II superconductor (see Fig. 1). The arrow marking the 'cooled-in-field' procedure passes through the melting line for the flux lattice. One thus freezes in the disorder existing in the vortex liquid. Once this disorder corresponding to field cooling is removed, it does not come back.

The phenomenon is not restricted to flux lines in a superconductor, but applies to elastic media more generally. The recrystallization effect has recently been predicted by Koshelev and Vinokur<sup>2</sup>, albeit for a two-dimensional system. They show that an elastic medium moving with sufficient speed against random pinning forces experiences a vibrational motion that mimics an annealing process through which disorder that was originally present in the medium is removed. Moreover, their computer simulations also produced the slight increase in disorder just before the onset of uniform flow. In a two-dimensional system, this increased disorder shows up as an increased density of edge dislocations.

Duarte *et al.* from the Centro Atomico in Bariloche, in collaboration with Yaron *et al.* (manuscript in preparation), very recently produced additional evidence for

this model. In their case the structure of the moving vortex lattice was probed at the surface of a single crystal of NbSe<sub>2</sub> by magnetic decoration — using fine iron particles to pick out the locations where flux lines leave the crystal — after abruptly switching off the driving current in order to fix the lattice configuration. By repeating the decoration experiment for a series of different driving currents, they were able to demonstrate the disordering of a slowly moving vortex lattice and the recrystallization when it moves quickly enough.

For such phenomena to be observable, it is important that the pinning is weak and fairly uniform. If strong fluctuations in the pinning are assumed, computer simulations made by Jensen *et al.*<sup>3</sup> have shown that a different kind of plastic flow occurs. This is usually characterized as partial flow, namely flow of a certain fraction of the vortex lattice through weak-pinning channels which develop amidst strong-pinning islands as the driving force is steadily increased. Such partial flow is believed to be commonly observed in strong-pinning, technically useful superconductors and was first described by Kramer<sup>4</sup> some 20 years ago.

Its weak pinning and good material properties have made NbSe<sub>2</sub> a favourite system for much of the present research in vortex physics. In contrast to the high-critical-temperature superconductors, which have  $H_{c2}$  of more than 100 tesla at  $T = 0$ , the entire field-temperature diagram is experimentally accessible. Near the melting line, as demonstrated by the noise and transport experiments of Bhattacharya and co-workers<sup>5</sup>, the behaviour of the lattice is plastic flow of the channel kind. The beautiful SANS and transport experiments of Yaron *et al.* now seem to show us that far below the melting line it is another kind of vortex lattice plasticity, plastic creep, that plays a crucial role in the depinning process at the critical current. □

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DAEDALUS

## Chemical skills

A TRUMPET player once turned up for a concert hopelessly drunk, yet astonished his colleagues by giving a virtuoso performance. "How can you play so well drunk?" they asked him. "Easy: I practise drunk!" he replied. This is 'state-dependent learning'. It has been blamed for many examination failures. Students who have learnt their material under the influence of caffeine and nicotine find themselves unable to recall it in the coffee-free, smoke-free examination hall.

At present, the effect is recognized only for addictive drugs. Rats taught a trick under the influence of librium, alcohol or barbiturate may lose the skill when they recover; and conversely. Daedalus cannot see why addictiveness matters. He reckons that many other drugs would show the effect if tested for it. He suspects that nostrums such as garlic, ginseng, kelp, cod-liver oil and so on work this way. Their faithful fans have carelessly allowed some crucial personal skill to come under the control of their nostrum: driving, perhaps, or getting up in the morning, or speaking with apparent authority. Reasonably enough, they feel that they cannot do without it.

Similarly, the many drugs discarded for their adverse effects on rats may merely have exerted state-dependent learning. They may have put the bemused rodents into a state forgetful of some crucial skill of rat life, such as finding the water supply or keeping their nests homelike, thus damaging their health. Alert for such effects, DREADCO rat psychologists are trawling anew the ranks of patent medicines and rejected drugs. Many of them may be safe but powerful agents of state-dependent learning.

The most promising finds will be tested for range and scope on human volunteers. Could a musician practise a dozen different instruments, each under the influence of a specific drug, with each skill unlocked again by its own key? Even the best 'learning drugs' may not be so specific. Even so, some may put the brain into an ideal state for learning music; others mathematics; others social skills or physical dexterity. The user will take the most appropriate pill for learning a given task. To exercise his skill he will simply take another, and then the whole topic will flood back in total recall, with no distraction. Education and adult learning will be transformed. A lecturing pill, a report-writing pill or a committee-chairing pill could smooth our climb up the learning curves of these tricky self-taught disciplines. And a passionate-lover pill, if taken regularly during our apprenticeship of this subtle art, should never let us down.

David Jones

# Dinosaur nests at the sea shore

SIR — Beach deposits of the Arenisca de Arén Formation<sup>1</sup> (southern Pyrenees, Maastrichtian, Upper Cretaceous) are rich in dinosaur eggs and bones, distributed over an area of about 15 km<sup>2</sup> along the northern flank of the Tremp syncline (Fig. 1). At one locality (Bastús, Lleida) we have estimated the presence of up to 300,000 eggs in a rock volume  $\approx 12,000$  m<sup>3</sup>, suggesting some kind of 'site fidelity'.

Dinosaur remains have frequently been reported from marine sediments. Efremov<sup>2</sup>

ing well-preserved dinosaur nests (Fig. 1). Farther down, the red layer passes into shore face sandstones with well-preserved, wave-induced sedimentary structures, forming part of a near-shore environment passing landward into mudflat deposits, occasionally dissected by small channels<sup>3</sup>. As the beach-ridge plain was built up to seaward (progradation), the dinosaurs nested in the exposed sandy sediment.

A huge number of egg shell fragments are scattered along the outcrop (Fig. 2).

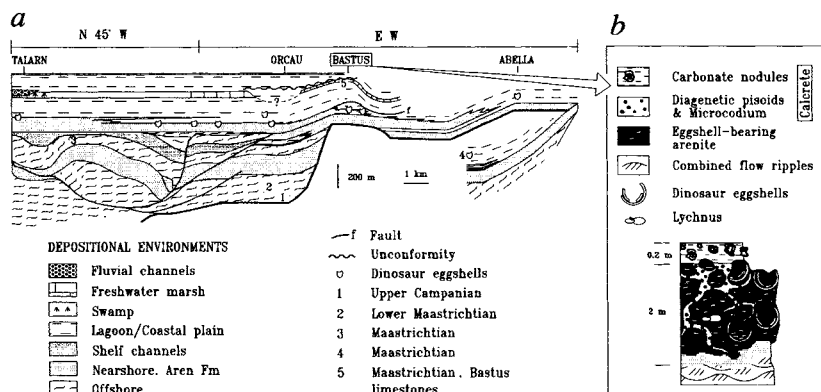


FIG. 1 a, Cross-section through the NW-prograding depositional sequence, Late Campanian and Maastrichtian in age, along the northern flank of the Tremp syncline (South Central Pyrenees, Spain). The succession formed under high terrigenous influx and relative sea-level rise. The sequence passes from coastal plain (Tremp Fm. p.p.) through strand-plain/barrier island (Arenisca de Arén Fm.) to offshore sediments. It overlies near-shore deposits of a similar sequence, which are affected by growth faults<sup>4</sup>. The dinosaur nests have been preserved mainly at the top of the Arenisca de Arén Formation. b, Log of the Bastús section. The matrix of the eggshell-bearing rock is a calcite-cemented, medium-to-coarse-grained arenite of mixed siliciclastic and carbonate grain composition. Carbonate grains comprise different types of worn skeletal fragments dominated by shallow-marine elements, intraclasts as well as dinosaur eggshell debris, and are generally affected by microbial micritization. This, along with the local preservation of relict dripstone and meniscus cement fabrics, suggests that lithification was initiated at the strand line (beach rock). Shoreline progradation then caused the incipiently cemented sand to become exposed to meteoric waters. Diagenesis in the freshwater phreatic environment involved selective leaching of initially aragonitic components and concomitant precipitation of new diagenetic calcite. Further diagenetic alteration before final burial of the sediment occurred in the meteoric vadose zone, as documented by the presence of a variety of petrographic features diagnostic of pedogenic calcrete development. c, Aspect of the outcrop.

suggested a marine habitat for some dinosaurs, although these were later interpreted as the remains of individuals that had been washed to the sea. Chanda<sup>3</sup> proposed a shallow-water marine environment for the dinosaur egg-bearing Lameta Formation of India, however, Shani *et al.*<sup>4</sup> conclusively argued for the continental (palustrine) origin of these sediments. Thus, the Arenisca de Arén egg sites represent the first unambiguous evidence of dinosaurs nesting at a sea shore.

The top of the Arén Formation at Bastús is 2-m-thick red sandstone contain-

Other fossils include large bone fragments; tiny smashed bones, probably belonging to young dinosaurs; a fairly complete skeleton of a small lizard and land snails (*Lychnus giganteus* Repelin). As the original disposition of some nests and bones are well preserved, post-depositional transport can be excluded as a primary cause of fragmentation. Rather, the eggs were destroyed by the animals' nesting and trampling activities and later by paedogenesis (Fig. 1). The nesting probably occurred in the unconsolidated sands of the beach ridge, in a short time interval between beach abandonment

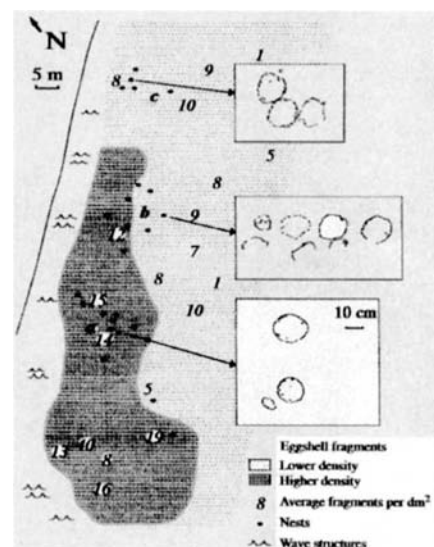


FIG. 2 Map illustrating nest distribution and eggshell density. The preserved nests, probably the last clutches, are in three main clusters. The distance between nests is 1–5 m (a), 4–6 m (b) and 3–8 m (c). The distance between some nests (1 m) is too short with respect to the presumed body size of the adult dinosaurs, suggesting that the nesting strategy is different from the colonial nesting behaviour described elsewhere<sup>6</sup>.

and early diagenesis (meteoric leaching, incipient lithification and paedogenesis).

The eggshell structure is tubospherulitic, and most of the preserved egg sections show subcircular contours. Some complete eggs from this locality, housed in the Freie Universität, Berlin, have a subspherical shape, with a diameter  $\approx 20$  cm. The outcrop has yielded the remains of 24 nests arranged in three clusters on a surface  $\approx 6,000$  m<sup>2</sup> (Fig. 2). Each nest contains 1–7 eggs, most of them having only two or three preserved eggs, representing portions of nests deposited in a hole dug in the sediment.

The Bastús nests are closely spaced but do not overlap (modal distance 2.5 m; Fig. 2). As the preservation state of the nests is generally good, some factor has precluded the destruction of previous clutches by newcomers. Some territorial behaviour may account for this feature. A high population density and/or scarcity of favourable substratum could explain the observed short distances between nests.

Using a 1-dm<sup>2</sup> grid in 20 randomly chosen areas (Fig. 2), we calculate that the eggshell material amounts to  $\approx 0.5\%$  of the total rock volume. Given the volume of the sandstone body (12,000 m<sup>3</sup>) and assuming an average egg size of 20 cm diameter and 1.45 mm shell thickness, we estimate

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that the lithic body contains the remains of some 300,000 eggs. This suggests that the area was a nesting ground, and that the dinosaurs may have returned to this same area during several reproductive seasons.

The Bastús egg site is the result of the nesting behaviour of a dinosaur population living very near the sea shore and may represent a local example of a widespread phenomenon in the Upper Cretaceous of the south-central Pyrenees.

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## Codon bias targets mutation

**SIR**—Serine is encoded by two sets of triplets (AGC and AGT (grouped as AGY) and TCA, TCC, TCG and TCT (grouped as TCN)). We have analysed the usage of the two types of codon in human immunoglobulin variable region (V) gene segments and have found a preference for AGY codons in the complementarity-determining regions (CDRs; particularly CDR1) and for TCNs in the frameworks (Fig. 1). This bias is not a peculiarity of human immunoglobulin V genes, but also occurs in mice (not shown) and *Xenopus*<sup>1</sup>.

Immunoglobulins undergo functional

diversification by somatic mutation, with nucleotide substitutions being introduced throughout the V-gene segment. Diversification is greatest in CDRs, the portions most strongly implicated in contacting antigen. We propose that the biased serine codon usage in immunoglobulins has evolved to help the somatic hypermutation machinery target these parts of the antibody molecule. Thus, mutations are targeted to residues that could yield increased affinity for antigen and away from places where they are more likely to destroy the structural scaffold. Consistent with this, the V genes of the T-cell receptor (TCR), where there is no evidence for functional diversification by somatic mutation, do not show a bias in their CDRs (Fig. 2).

During maturation of the antibody response, the V regions in those B lymphocytes selected by the initial antigen challenge contain many nucleotide substitutions. Cells expressing antibodies with improved binding characteristics are then selected from the population of somatically mutated daughter cells. The nucleotide substitutions are not targeted randomly. In mouse variable-region  $\kappa$ -chains ( $V_{\kappa}$ s), for example, there is intrinsic favouring<sup>2</sup> of CDR1, and hotspots of mutation have been identified that are intrinsic to the mutational process<sup>2,3</sup>. The consensus  $[^A/G G C/T ^A/T]$  has been proposed<sup>4</sup> as a preferred target for mutation and, indeed, the most striking of the hotspots are often associated with AGY serine codons<sup>2,3</sup>. Thus, the preponderance of AGY over TCN serines in the CDRs of germline V genes (and the converse in frameworks) suggests that the DNA sequence of germline V genes has evolved in response to selection for appropriately targeted mutability.

There are some significant exceptions to this biased codon usage. In germline  $V_{\kappa}$ s, a

FIG. 1 Distribution of TCN and AGY serine in immunoglobulin V segments. *a*, *b*, Sequences of functional human  $V_H$  genes (kindly provided by G. Cook and I. Tomlinson<sup>7</sup>) and  $V_{\kappa}$  genes<sup>8</sup> have been grouped into families and, at each serine position, the number of family members containing a TCN codon is depicted above the line and an AGY codon below. The name of each family and the number of its members are given. CDRs are shaded. *c*, The ratio of serines encoded by AGY triplets to those encoded by TCN triplets is given for CDR1, CDR2 and the framework portions (in FR1, FR2 and FR3 are summed together) of human immunoglobulin (Ig)  $V_H$  and  $V_{\kappa}$ , and TCR  $V_{\alpha}$  and  $V_{\beta}$  segments. This compilation has been made by combining all Vgene sequences for each locus. Using a large sample test for a binomial distribution, the AGY/TCN ratios in the  $V_H$  CDR1 and CDR2 and in the  $V_{\kappa}$  CDR1 are significantly skewed ( $p < 0.03\%$ ) from the ratio (0.64) expected from the GenBank protein database<sup>9</sup>. There is slight inverted skewing of the AGY/TCN ratio in the case of the TCR CDRs, although this is of much less statistical significance.

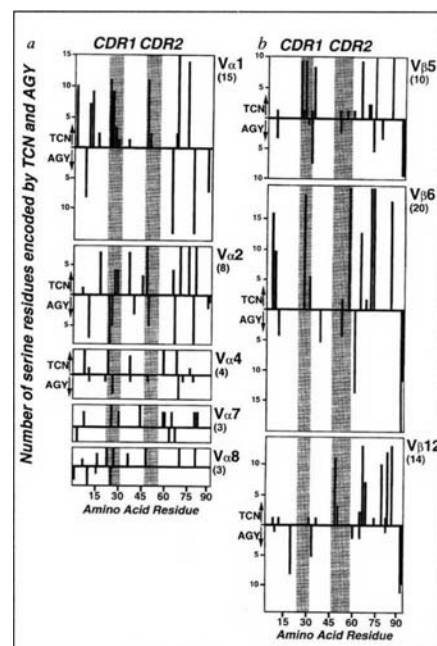
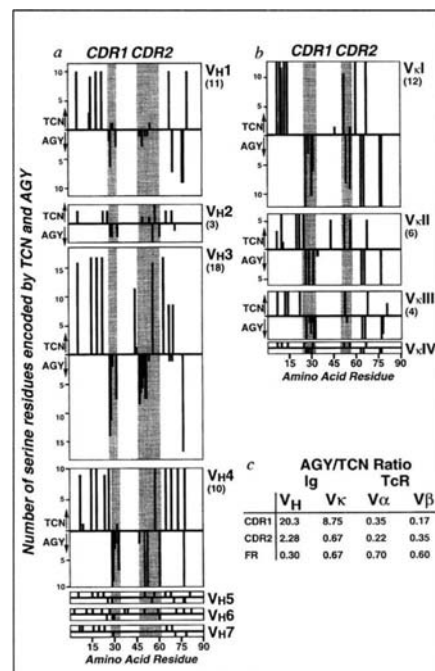


FIG. 2 Distribution of TCN and AGY serine in TCR V segments. Sequences of human TCR  $V_{\alpha}$  and  $V_{\beta}$  genes (classified as in ref. 10) have been grouped into families and the serine codon usage displayed as in Fig. 1. Only the largest TCR Vgene families were used for the analysis.

TCN serine (position 52) is conserved in CDR2 presumably because it contributes to the structural integrity of the CDR loop<sup>5</sup>. On the other hand,  $V_{\kappa}$  framework position 77, a conserved AGY in human and mouse germline  $V_{\kappa}$ s, is one of the intrinsic mutational hotspots located outside a CDR<sup>2</sup>. There is also a preference for serine AGY codons in positions at the 3' end of TCR  $V_{\beta}$  genes flanking the heptamer/nonamer recombination signals which may reflect a role in the gene rearrangement process.

It has been noted that serine AGY codons are more mutable than serine TCNs in protein coding sequences of humans and mice. This observation parallels the mutability of V-gene serine codons during somatic hypermutation and is consistent with them being common features of the molecular mechanism of evolutionary and somatic mutation.

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# Informed by nature's light

Arthur Zajonc

**Color and Light in Nature.** By David K. Lynch and William Livingston. Cambridge University Press: 1995. Pp. 254. £40, \$69.95 (hbk); £17.95, \$29.95 (pbk).

**Optics in the Age of Euler: Conceptions of the Nature of Light, 1700–1795.** By Casper Hakfoort. Cambridge University Press: 1995. Pp. 243. £35, \$64.95.

THE subject of light warrants a wide range of treatments, and these two books exemplify the extremes between which such treatments can fall. At one end of the spectrum is *Color and Light in Nature*, a beautifully illustrated popular account of atmospheric phenomena written by David Lynch and William Livingston, both of whom are US astronomers with a fine eye for colour in the sky. At the other end lies Casper Hakfoort's meticulous research monograph on the conceptions of light between 1700 and 1795. Both volumes are worthy additions to the corpus of works on light.

Lynch and Livingston pick up where Minnaert left off in his classic *The Nature of Light and Colour in the Open Air*. They take the reader through hundreds of light and colour phenomena visible in the sky overhead, ranging from the straightforward to the exotic. Each account is succinct and lucid, illustrated by both diagrams and photographs, some stunning in their beauty. A distinguishing feature of their account is a systematic attempt to give clear scientific explanations for each effect, including appropriate graphs and tables, while avoiding the underlying mathematics. Often the treatment is straightforward, but in a surprising number of cases one can make reasoned assertions only, which the authors are careful to frame as such. The balance of description and physical explanation is excellent. Many teachers as well as a good many naturalists will find the book to be a highly useful and comprehensive treatment of a beautiful subject, one that can be used to enliven dry classroom discussions of optics, light and colour.

My one complaint is that the authors do not give enough emphasis to the role of the human visual system in producing some of the effects. For example, in their treatment of coloured shadows, they include in the analysis the role of the incident light and the object but neglect the psychological dimension. It is well known that coloured shadows are largely due to a psychological effect sometimes termed chromatic adaptation. The entire book is concerned with seen colours, and these arise not only through the physical interaction of light with air and water, but also depend on the complex action of the eye and mind of the observer.

In *Optics in the Age of Euler*, Casper

Hakfoort provides us with a careful, one might even say cautious, study of the various conceptions of light prevalent during the Enlightenment. This is a forthright historical presentation of the major (and not so major) theories of light, proceeding chronologically from Newton and Descartes through to Euler and the subsequent reception of his wave theory. Hakfoort strives throughout to avoid historical condensation by giving detailed, nuanced treatments of specific ideas held by specific individuals. One will find no sweeping generalizations here, but only the measured voice of careful textual analysis (often in the original language). The author summarizes clearly the ideas of each person, noting differences and commonalities before moving on to the next one.

For example, the usual simplification of 'wave versus particle' is avoided. At the beginning of the eighteenth century the debate was, rather, between mecha-

nistic accounts of light, whether Newton's or Descartes', and those derived from Aristotle that held that qualities are not derivative but have ontological status in themselves. The antagonism between emission theories and 'medium' theories arose only gradually, coming into clarity in Euler's important work *Nova theoria* (1746) which presented the first serious medium theory of light. Surprisingly, Hakfoort shows that many in Germany persisted in holding to a wave theory until photochemical experiments came onto the scene. Until this point, little originality is apparent in the book, but in his epilogue Hakfoort advances, again cautiously, a historiographical suggestion that I find convincing. Science from 1700 on is usually divided (following T. S. Kuhn) into mathematical and empirical branches. Hakfoort argues that this two-fold division misses the important stream of natural philosophy still active in the eighteenth century. For Descartes, Euler and others, the issue is not only one of mathematical formalism, nor of data alone, but what light really is. One might go even further than Hakfoort and ask whether science even today has given up the longing to know the true nature of things. □

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## World of vision at a glance



EDWARD Hopper's *Sun in an Empty Room* (1963). The picture is reproduced in *Perception* by Irvin Rock, one of two books on the visual sciences that are published in the Scientific American Library series and which are now available in paperback. The other volume is the highly acclaimed *Eye, Brain, and Vision* by David H. Hubel. Both books are profusely illustrated and will appeal to a wide audience, including students of art, psychology and neuroscience. W. H. Freeman/Scientific American Library, \$19.95, £14.95 each.



# Chemical cosmography

Stephen Mason

## The Periodic Kingdom: A Journey into the Land of the Chemical Elements.

By Peter Atkins. Weidenfeld and Nicholson/BasicBooks: 1995. Pp. 161. £9.99, \$20.

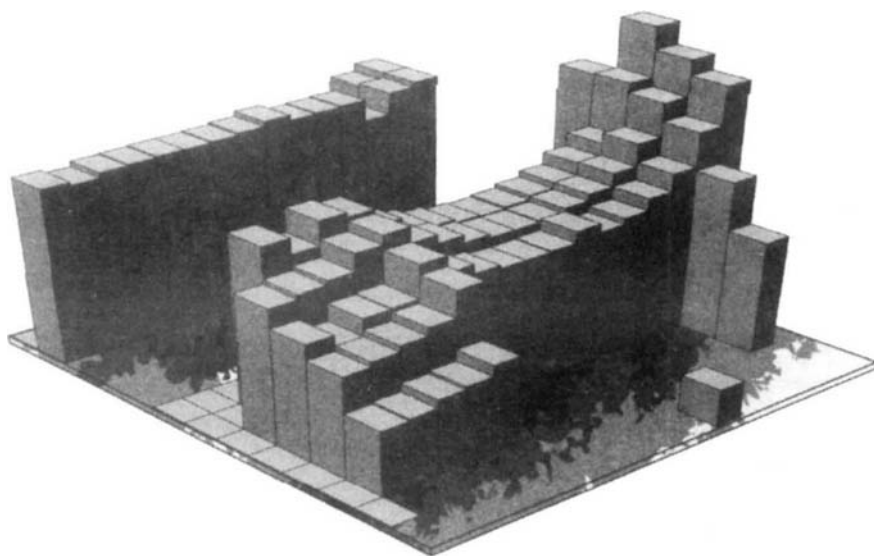
*The Periodic Kingdom* was inspired by the opening of Somerset Maugham's *The Vessel of Wrath*, in which the author, leafing through a navigational guide to the Yangtse Kiang, envisages the fertile lands and bustling life along the length of the river. Similarly, Peter Atkins uses the periodic table to guide us through an imaginary land of ordered regions, each corresponding to one of the hundred or so chemical elements.

From overhead, a panorama extends from the northern island of hydrogen to

falls from east to west, and from north to south, according to the feature portrayed.

The history of the Kingdom describes how the chemical elements came to be discovered, and how the cartographers mapped related regions. The mapping culminated with Mendeleev, who produced a definitive chart, including the location and attributes of regions so far unknown, to guide future explorers. One of these explorers, Lecoq de Boisbaudran, supposedly named the element gallium, which he discovered, after his native Gaul, but probably celebrated himself (*Gallus gallus*, the cock).

Atkins's journey concludes with an account of the Periodic Kingdom's government and institutions. These are grounded upon the discovery of the nuclear atom and the great invention of quantum mechanics. The ordering of the orbital electron around the nucleus by the laws of quantum mechanics explains the old geography, and the singular trends in atomic properties, in new terms



Physical geography — the Periodic Kingdom plotted in terms of the diameters of atoms, viewed from the northeast corner. The diameters are based on the lengths of the bonds the elements form, so the noble gases are not ascribed values here because they do not form bonds. Picture taken from *The Periodic Kingdom*.

distant regions beyond uranium in the south, passing through the western desert of the transition elements. The desert constitutes an isthmus joining the western rectangle of alkali metals and alkaline earths to the eastern rectangle containing the non-metals and metalloids, with a bridge linking the isthmus to the off-shore southern island of lanthanides and actinides. An inspection of the products of the various regions reveals that carbon, with its wide-ranging liaisons and fecundity, is king of the Periodic Kingdom. Probes of such properties as the atomic volume, or the ionization energies of the elements, map out secondary terrains with singular rises and

— the s-, p-, d- and f-shells. These laws also characterize the nature and the shapes of the molecular liaisons, which are as indefinitely numerous as the literature constructed from the letters of the alphabet.

*The Periodic Kingdom* requires no previous knowledge of chemistry. All concepts are introduced with clarity illuminated by witty charm. The book makes an outstanding contribution to the public understanding of science. □

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## Windows 95

As an antidote to the hype surrounding last week's worldwide launch of Windows 95, it is worth delving into the second edition of *The Computer Contradictionary* by Stan Kelly-Bootle (MIT Press, £14.95 (pbk)) for a few irreverent definitions:

**windows** *n.* Also capitalized

**Windows** as presumed (disputed) trademark of MICROSOFT. *Derog. form* **Windoze**. Conspicuous tessellation promoted by the purveyors of RAM and MIPS. See also GATES, WILLIAM; GUI; X WINDOWS.

⇒The Windows literature tells us "... whenever an application creates a window, Windows sends that window a WMCREATE message. This is certainly reasonable, since being created is after all an event that might be of interest to the window" (Durant, Carlson, and Yao, *Programmer's Guide to Windows* [Alameda, Calif.: Sybex, 1987], p. 151).

One is tempted to add that, prior to creation, the window is incapable of showing interest or disinterest in anything at all. I detect a neo-Cartesian autoepistemic syllogism here: "I (window speaking) have been told that I am; I hear this interesting message; therefore I am."

**Microsoft Corporation** also called **MS**; *derog. Microsloth*; *laud. The IBM of the 1990s*. The dominant marketing force in OS and applications software, beyond the slings and arrows of bankrupted rivals' mockery; also well-beyond any interference from ACLU, ANSI-ISO, FCC, all Monopolies' Commissions, GATT, NATO, and the UN Security Council. See also DINOSAURS; MS-DOS; GATES, WILLIAM; IBM; MAW; UNDOCUMENTED; WINDOWS.

⇒As with IBM's dominance in the 60s-70s, the supremacy of Microsoft in the 80s-90s proves that innovation and sworn delivery promises should never be allowed to interfere with marketing. Another depressing IBM-MS parallel is that defeated competitors invoke conspiracies and scream MONOPOLY.

**Gates, William** (member FDIC, aka **Bellevue Billy**) Founder/CEO of Microsoft Corporation, and youthfully, richly immune to jealous satire. As the second wealthiest U.S. billionaire, he tries harder. See also MAW; UNDOCUMENTED; WINDOWS.

⇒Bill sensibly disputes the *Forbes*'s annual wealth tables. A minor fluctuation in Microsoft share values can increase or decrease his net worth by untold millions. We, *los decamisados*, must count our blessings.

## More than a seafood platter

Robert A. McCall and Robert M. May

**Fishbase: A Biological Database on Fish (CD-ROM and User's Manual).** Edited by R. Froese and D. Pauly. International Center for Living Aquatic Resources Management, Manila, Philippines: 1995. \$95.

WITH more than 30,000 publications listed annually in the *Aquatic Sciences and Fisheries Abstracts*, knowledge of fish and fisheries is increasing at a prodigious rate. The difficulty lies in making this knowledge accessible to a wide range of potential users. The problem is particularly severe in many developing countries, where fisheries may be central to the well-being of the population yet up-to-date information on their proper management may be unavailable.

Motivated by these concerns, workers at the International Center for Living Aquatic Resources Management in Manila, Philippines, have compiled a new fin-fish database on CD-ROM. Called *Fishbase*, it is the most comprehensive database of its kind, covering the biology, distribution and taxonomy of roughly 12,500 species of fish — about half the estimated total number of extant fish species. What is more, the database includes all commercially important species, which adds to its usefulness in fisheries management. Much of the information comes from the UN Food and Agricultural Organization's 'SPECIES-DAB' lists, and the project itself is funded largely by the European Union.

Central to the database is the 'species table', which contains basic information about each species. From this table one can access other tables which cover in detail nine main areas of piscine biology: taxonomy, global distribution, population dynamics, trophic ecology, reproduction, ichthyoplankton, morphology and physiology, genetics and aquaculture. The amount of information is enormous, with data on everything from swimming speeds, to diet, to genetic variability. Inevitably, the information is sometimes incomplete, but the authors are committed to upgrading the database as new data become available — an updated version, with lots of improvements and additional material, is in fact already on its way. Publication of the database will itself highlight gaps in our knowledge and stimulate new research.

The authors have, however, ambitiously aimed to provide more than a well-posted gateway into a vast primary literature and an analytical and searchable summary. For one thing, they see *Fishbase*



SILVER shoal — bigeye trevallies (*Caranx sexfasciatus*) are open-water predators that chase and herd shoals of smaller fish, sometimes driving them towards the surface and causing them to panic so that they can pick off their prey. Picture taken from *The Living Sea: A Photographic Exploration of Life in the Sea* by Linda Pitkin. Fountain, £24.95.

as being useful to undergraduate and postgraduate students, offering a comprehensive overview of fish biology (complete with pretty pictures illustrating adults, larvae and sexual dimorphism) along with data capable of fuelling highly specialized projects. Their avowed aim of producing a database that is both "global and deep" seems to have been achieved.

Perhaps with still younger users in mind, the package includes something called FishQuiz. Pictures are displayed on the screen in a random order and the user is invited to choose, from a multiple choice, which family, genus and species are represented. There are, praise be, no sound effects or halls of fame.

*Fishbase* is currently of limited value to studies of biodiversity, mainly because of the lack of adequate records for many species. But the addition of an accepted phylogeny, if and when one becomes available for fin-fish, would turn it into a powerful tool for evolutionary biologists; as it stands, the collection of data on life-history and population parameters, together with existing phylogenies, will surely find use in comparative studies.

*Fishbase* is contained on a single CD-ROM and comes with a detailed manual on how to use it and where to find the data tables. It requires a 486 DX2

machine or better, with 8 (but preferably 16) megabytes of random access memory, and operates from Windows (version 3.1 or later). A good quality VGA display is recommended for the colour pictures. *Fishbase* is impressive to use: the operating system is clearly laid out so that users can easily move from one dataset to another. Distribution data can be displayed as a WinMap plot (a minor fault is that this is shown as countries on a map where a given species has been reported or collected rather than as a plot of distribution *per se*) and a built-in glossary contains definitions of more than 1,000 terms.

In short, *Fishbase* draws together and makes accessible a huge amount of information about fish and fisheries, much of which was previously buried in the 'grey literature' of reports from fisheries institutes or working parties. By summarizing what we already know and indicating future directions for study, it will be useful for both researchers and students. Perhaps most important, and certainly closest to the authors' hearts, it will benefit developing countries, where the lack of comprehensive libraries is often keenly felt. □

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# Making sense of bodies

Simon Conway Morris

**Animal Evolution: Interrelationships of the Living Phyla.** By Claus Nielsen. Oxford University Press: 1995. Pp. 467. £55, \$95 (hbk); £25, \$49 (pbk).

WHAT is it about metazoan studies that paralyses initiative and reduces the student to stunned inertia? Libbie Hyman's masterly series *The Invertebrates* finally ground to a halt in its sixth volume with her valedictory remark: "What with advanced age (78) and concomitant loss of strength and energy, it has become a physical impossibility for me to continue". Check some of the recent massive textbooks on animal biology; it cannot be long before the US obsession with litigation warns would-be readers against "hernia and sudden rupture". Here in contrast is a book that aims to encompass this vast field in a manageable style. How well has it succeeded?

Its strengths are a clear and engaging style exemplified by a series of superbly concise descriptions of the phyla, 31 of which are recognized. These are complemented by excellent illustrations, albeit the minimum necessary. The volume belongs on every biologist's bookshelf. Its low price means that generous individuals can donate a copy to their colleagues in molecular biology for whom a slightly wider acquaintance with whole-organism zoology might be salutary.

But will this book prove to be the banner in the much needed renaissance of the study of metazoans and their phylogeny? Here reservations begin to emerge. The book is underpinned by two main precepts: the cladistic approach and the fundamental importance of embryology and larval development for our understanding of Metazoa. It remains, nevertheless, a curious fact that it is extraordinarily difficult to decide which characters are phylogenetically informative rather than convergently acquired, presumably because of similar adaptive constraints. Writing of one larval structure found in several phyla, Nielsen opines that: "To me it appears very improbable that the highly characteristic ciliary bands... should have developed convergently". But why not? Further on in the book we read that another character (gap junctions) "must be regarded as a remarkable example of convergent molecular evolution". Such declarations, however intelligent, are really acts of faith (the few theologians who read *Nature* may permit themselves a wry smile). Here a very interesting opportunity seems to have been missed, because this book is a gold mine for examples of evolutionary convergence. A striking example is

the tri-radiate pharynx. In this case functional constraints are probably the overriding factor. The point to make, however, is that there are limits to design, and one way in which metazoan studies may progress is by an explicit exploration of this field.

The most glaring omission is the evidence from molecular biology. Nielsen simply states that he wishes to use morphological data alone. This is his privilege, but he does no service either to the next generation of investigators or to the important contributions that molecular data are now making to metazoan phylogeny. It should be self-evident that science flourishes in a state of tension: the nagging doubt that the bits are still not falling into place. For example, in terms of metazoan relationships, there is a clear dichotomy between placing the lophophorates in the deuterostomes, the orthodox line that Nielsen follows, and the molecular (and palaeontological) data that regard them as mainstream protostomes. Somebody has got to be wrong. Nor need there be endless divergences in opinion. Nielsen's belief "that the tracheates are a specialized group of terrestrial crustaceans" matches very well the latest views from the molecular camp (*Nature* 376, 163-167; 1995). But where things really begin to unravel is when we consider the basic developmental instructions for complex organ systems such as eyes in arthropods or neural tubes and mouths in deuterostomes. Nielsen wrestles with what features in phyla are genuinely homologous, but the story emerging from molecular biology is that what may look very different in anatomical terms can be founded on a basically identical genetic architecture.

Eyebrows will be raised elsewhere, but we should not automatically rush to condemn. Thus Nielsen continues to ally the ectoprocts and entoprocts. This idea has won few adherents, but supposing the molecular sequences reveal a close relationship? Could somebody oblige? Courageously, Nielsen locates the ctenophores close to the deuterostomes. Every textbook I can think of places them about as far away as it is possible to go, near the cnidarians. Such molecular data as there are support the latter alternative, but ctenophores remain very puzzling.

These comments should not detract from what is an excellent book. The men in suits will no doubt be offended by Nielsen's forthright style. I am not sure I would care for my life's work to be dismissed as "mere fantasy", as he effectively does of one worker, but it is only by invigoration, argument and enthusiasm that the real problems in understanding the metazoans will be confronted. □

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## Children's Books

*Nature* plans to publish on 16 November a supplement in which children's books will be reviewed. The supplement will cover science books and software for children of all age groups.

Publishers are invited to send suitable material for consideration, taking note of the following criteria:

■ Only books and software issued in 1995 will be considered;

■ Books and software dealing with any aspect of science, technology, medicine or natural history are eligible (including encyclopaedias, dictionaries and games), although those specifically tailored to mainstream school curricula are excluded;

■ The main language used must be English;

■ If possible, cross-platform software (both Macintosh and PC) should be provided.

Deadline for submission is 22 September.

Publications for review should be sent, together with details of price and availability, to Peter Tallack, Book Review Editor, *Nature*, 4 Crinan Street, London N1 9XW, UK (tel: +44 (0)171 843 4567; fax: +44 (0)171 843 4596/7; e-mail: p.tallack@nature.com).

## New in paperback

**Exploring Evolutionary Biology: Readings from American Scientist** edited by

Montgomery Slatkin. W. H. Freeman, £18.95. A cast of eminent contributors tackle such topics as "Who killed the dinosaurs?" (William Glenn), "Fisher's microscope, or the gradualist's dilemma" (Keith Stewart Thomson), "Evolution and the triumph of homology, or why history matters" (Stephen Jay Gould), "Untangling the evolution of the web" (William A. Shear), "Female choice in mating" (Meridith F. Small), "Naked mole rats" (Rodney L. Honeycutt) and "The evolution of primate behaviour" (Alison Jolly).

**A Primer of Ecology** by Nicholas J. Gotelli. W. H. Freeman, £13.95. A clear and concise supplementary text for introductory courses.

**Meanings of Sex Differences in the Middle Ages: Medicine, Science, and Culture** by Joan Cadden. Cambridge University Press, £14.95, \$18.95. A

scholarly enquiry into how biological notions about reproduction and sexual impulses and experiences intersected with ideas about the social roles of men and women, the purpose of marriage, the road to salvation and so on.

**Time, Space and Things** by B. K. Ridley (3rd edn). Canto (Cambridge University Press), £5.95. Sets out to survey in simple, non-mathematical terms what physics has to say about the fundamental structure of the Universe. Now updated to cover strings, imaginary time and chaos. Provides a lucid introduction for nonspecialists while offering a fresh perspective to students of physics.

# Protein tyrosine kinase PYK2 involved in $\text{Ca}^{2+}$ -induced regulation of ion channel and MAP kinase functions

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Departments of Pharmacology<sup>\*</sup> and Physiology<sup>†</sup>, New York University Medical Center, 550 First Avenue, New York, New York 10016, USA  
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**The protein tyrosine kinase PYK2, which is highly expressed in the central nervous system, is rapidly phosphorylated on tyrosine residues in response to various stimuli that elevate the intracellular calcium concentration, as well as by protein kinase C activation. Activation of PYK2 leads to modulation of ion channel function and activation of the MAP kinase signalling pathway. PYK2 activation may provide a mechanism for a variety of short- and long-term calcium-dependent signalling events in the nervous system.**

PROTEIN tyrosine kinases are important for the control of cell growth and differentiation<sup>1,2</sup>. Several protein tyrosine kinases are highly expressed in the central nervous system<sup>3</sup>, and there is evidence that protein phosphorylation is important for regulation in the nervous system. Neurotrophic factors that control the differentiation and survival of different types of neuronal cells mediate their biological effects by activation of cell surface receptors with intrinsic protein tyrosine kinase activity<sup>4</sup>. Furthermore, protein phosphorylation is a key regulatory mechanism for membrane excitability and ion channel function. It has been shown that ion channels are subjected to phosphorylation that alters their functional properties<sup>5,6</sup>. Most of the currently available information is restricted to modulation of ion channel functions by serine/threonine kinases such as protein kinase C (PKC), cyclic AMP-dependent protein kinase and calmodulin-dependent kinase<sup>7-10</sup>. It has been shown that PKC can regulate the action of a variety of ion channels, including voltage-gated potassium channels<sup>10</sup>, voltage-dependent sodium channels<sup>7</sup> and the nicotinic acetylcholine receptor<sup>3</sup>. Tyrosine phosphorylation also has a regulatory role in the action of specific ion channels, such as the *N*-methyl-D-aspartate (NMDA) receptor<sup>11</sup> and a delayed rectifier-type potassium channel<sup>12</sup>. In the latter example, inhibition of potassium currents is mediated in part by tyrosine phosphorylation of the channel protein by an unknown protein tyrosine kinase<sup>12</sup>. Thus it is clear that the action of protein kinases and phosphatases is crucial for the control of proliferation, differentiation and survival of neuronal cells, as well as for the regulation of neuronal excitability, plasticity and excitotoxicity.

## Cloning of PYK2

To isolate Grb2-binding proteins, we have used the Grb2 adaptor protein as a specific probe for screening expression libraries. One of the cloned proteins encoded a protein tyrosine kinase with a proline-rich region that can bind *in vitro* to the SH3 domains of Grb2 (J. Ureña and J.S., unpublished results). This protein was termed proline-rich tyrosine kinase 1 (PYK1). Comparison of the amino-acid sequence of PYK1 to other tyrosine kinases indicated that PYK1 is related to the Ack protein tyrosine kinase<sup>13</sup>, and that it represents a member of a new class of cytoplasmic protein tyrosine kinases.

To isolate kinases related to PYK1, we applied the polymerase chain reaction (PCR), using degenerate oligonucleotide primers derived from the PYK1 sequences in conserved motifs of cata-

lytic domains of PTKs<sup>14</sup>. Figure 1a shows the complete amino-acid sequence of a protein tyrosine kinase that was isolated from a human brain complementary DNA library and termed PYK2. The open reading frame of PYK2 encodes a protein of 1,009 amino acids, containing a long amino-terminal sequence of 425 amino acids followed by a protein tyrosine kinase domain, two proline-rich domains (29 and 23.3% proline, respectively), and a large carboxy-terminal region. The kinase domain of PYK2 (Fig. 1a, box) contains several sequence motifs conserved among protein tyrosine kinases, including the tripeptide motif DFG that is found in most kinases, and a consensus ATP-binding motif GXGXXG followed by an AXK sequence 17 amino-acid residues downstream<sup>14</sup>.

Comparison of the amino-acid sequence of the kinase domain of PYK2 with other protein tyrosine kinases showed that the kinase core of PYK2 is most similar to the protein tyrosine kinase domains of Fak, Fer, Her4 and Abl (Fig. 1b). In addition to the sequence homology in the kinase domain, the flanking sequences and the overall structural organization of the PYK2 protein are most similar to those of Fak<sup>15</sup>. This suggests that PYK2 and Fak belong to the same family of non-receptor protein tyrosine kinases.

We next examined the expression pattern and tissue distribution of PYK2 by northern blot and *in situ* hybridization analyses. Northern blot analysis of multiple human tissues demonstrated that a 4.5-kilobase (kb) PYK2 transcript is most abundant in the human brain, with a low level of expression detected in the kidney (data not shown). *In situ* hybridization analysis showed that PYK2 was expressed in discrete populations of neurons within the rat brain, with the highest levels of mRNA seen in the hippocampus, dentate gyrus and olfactory bulb (data not shown). These results are consistent with northern blot analysis of messenger RNA isolated from various human brain sections (data now shown).

To characterize the PYK2 protein, specific antibodies were raised in rabbits against a PYK2 fusion protein. These antibodies precipitated a protein from PC12 cells, as well as from transfected NIH 3T3 cells, that migrated in SDS gels with an apparent relative molecular mass ( $M_r$ ) of 112K (data not shown).

## Activation of PYK2

Because PYK2 is highly expressed in the central nervous system and PC12 cells, we examined the effect of a variety of neuronal stimuli on the phosphorylation state of PYK2. In these experiments, PC12 cells were treated with agonist, lysed, and immuno-

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**FIG. 1** Primary structure of human PYK2 protein tyrosine kinase. **a**, Deduced amino-acid sequence of human PYK2 from cDNA clones. The tyrosine kinase domain is highlighted by a dark shaded box. Two proline-rich domains in the C-terminal region are boxed with light shading. Amino-acid residues are numbered on the left. **b**, Comparison of the amino-acid sequence of the catalytic domain of PYK2 with the four most related human protein tyrosine kinases demonstrated 61%, 43%, 40% and 41% sequence identity between PYK2 and Fak, Fer, HER4 and Abl, respectively. The homology between PYK2 and Fak extends beyond the catalytic domain with 42% and 36% amino-acid identity in the N-terminal and C-terminal regions, respectively.

**METHODS.** Total RNA from rat spinal cord was used to prepare cDNA using the reverse transcriptase of Molony murine leukaemia virus ('Superscript', BRL) according to the manufacturer's protocol. The cDNA was amplified by PCR utilizing degenerate oligonucleotide primers

corresponding to conserved tyrosine kinase motifs from subdomains TK6 and TK9 of PYK1 (J. Ureña and J.S., unpublished results); (the sense and antisense primers correspond to amino-acid sequences IHRDLAARN and WMFGVTLW, respectively). The PCR was performed under the following conditions: 1 min at 94 °C; 1 min at 50 °C and 1 min at 68 °C for 35 cycles. Amplified DNA was subcloned and sequenced, resulting in identification of a novel tyrosine kinase termed PYK2. A  $\lambda$ gt10 human fetal brain cDNA library (Clontech) was screened with a  $^{32}$ P-labelled probe derived from the PCR clone corresponding to rat PYK2. Four overlapping clones were isolated and their DNA sequence was determined on both strands using series of oligonucleotide primers. The 3,414-bp consensus sequence contains a single open reading frame of 3,027 nucleotides

preceded by a 105-nucleotide 5'-untranslated region. The complete nucleotide sequence was deposited in GenBank (accession no. 33289). Amino-acid sequence comparisons were performed using the Smith-Waterman algorithm of MPSRCH (IntelliGenetics) on a MasPar computer.

precipitated with anti-PYK2 antibodies, followed by SDS-PAGE (polyacrylamide gel electrophoresis) analysis and immunoblotting with phosphotyrosine-specific antibodies.

Stimulation of PC12 cells with carbachol induces strong tyrosine phosphorylation of PYK2 (Fig. 2a). Because carbachol can activate both the nicotinic and muscarinic receptors<sup>16</sup>, we explored whether activation of both cholinergic receptor subtypes leads to tyrosine phosphorylation of PYK2. Pharmacological analysis with either subtype-specific agonists, muscarine and dimethylphenylpiperazine (DMPP), or subtype-specific antagonists, atropine and mecamylamine (Fig. 2a) indicated that activation of PYK2 by carbachol is mediated by means of the nicotinic acetylcholine receptor. The phosphorylation of PYK2 in response to carbachol is very rapid; 5 s after applying carbachol to the cells, PYK2 became phosphorylated on tyrosine residues. Elimination of extracellular calcium by EGTA completely blocked this agonist-induced tyrosine phosphorylation of PYK2 (Fig. 2a, lower panels), indicating that calcium influx is required for carbachol-induced PYK2 phosphorylation.

Stimulation of the nicotinic acetylcholine receptor induces membrane depolarization by cation influx through the ion channel pore. This depolarization opens voltage-gated calcium channels, resulting in a large influx of calcium into the cell. We therefore checked whether membrane depolarization induced by increasing the extracellular concentration of potassium ions would give the same effect on PYK2 tyrosine phosphorylation. Depolarization of PC12 cells with 75 mM KCl induced rapid tyrosine phosphorylation of PYK2 (Fig. 2b). The omission of calcium from the extracellular medium completely abolished this PYK2 tyrosine phosphorylation, indicating that activation of PYK2 is due to calcium influx rather than membrane depolarization *per se*. To explore this possibility further, we examined the

effect of a calcium ionophore (A23187) on PYK2 activation: PYK2 is phosphorylated on tyrosine residues after incubation with A23187 (Fig. 2b, right). Elevation of intracellular calcium in response to a variety of stimuli therefore causes tyrosine phosphorylation of PYK2. Moreover, the intrinsic protein tyrosine kinase activity of PYK2 was stimulated by carbachol or KCl, leading to strong tyrosine phosphorylation of the exogenous substrate poly(Glu-Tyr) (4:1) (Fig. 2c).

## Role of G-protein-coupled receptors

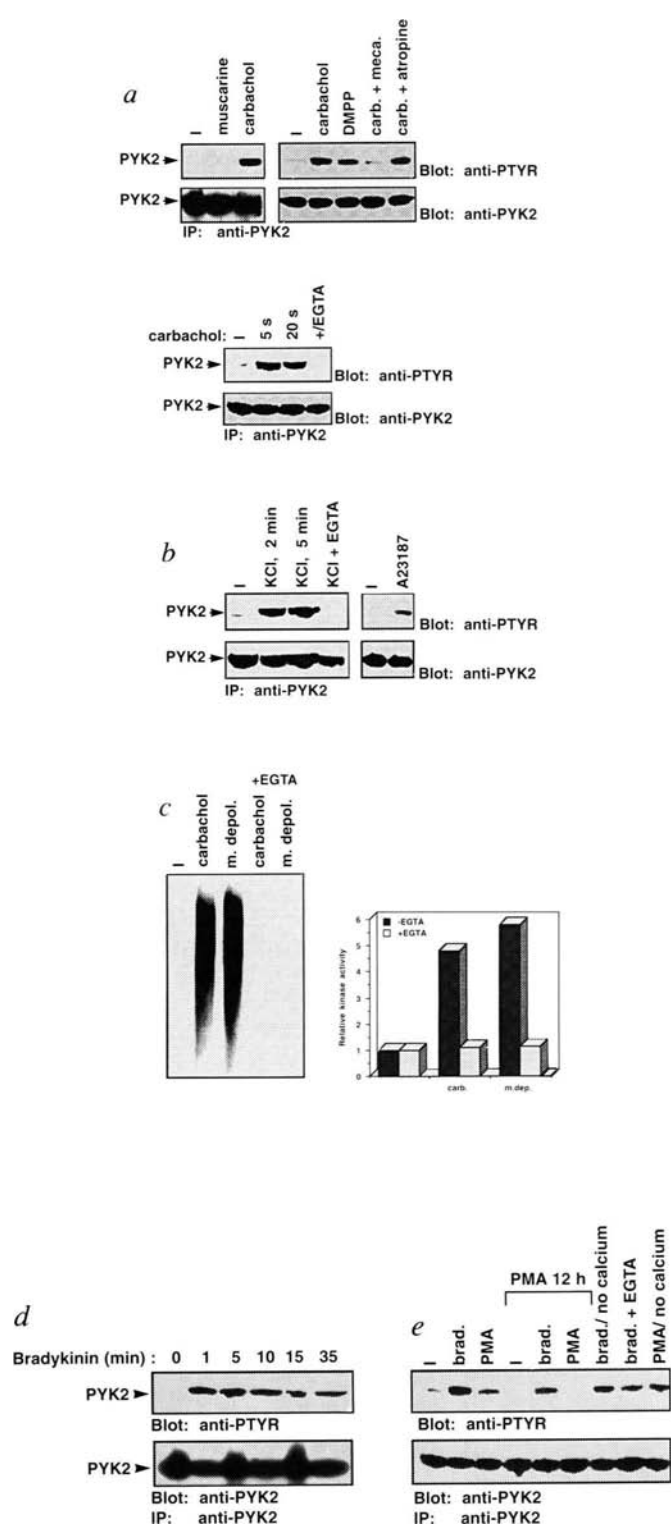
The neuropeptide bradykinin elicits a wide range of biological responses in various cell types and tissues<sup>17</sup>. In PC12 cells, bradykinin activates a G-protein-coupled receptor that stimulates the production of diacylglycerol (DAG) and inositol-1,4,5-trisphosphate (Ins(1,4,5)P<sub>3</sub>) by activation of phospholipase C<sup>18</sup>. DAG activates the Ser/Thr protein kinase C (PKC), whereas Ins(1,4,5)P<sub>3</sub> increases cytosolic calcium levels by triggering the release of calcium from intracellular stores<sup>19</sup>. Because bradykinin increases intracellular calcium concentration, we analysed its effect on the phosphorylation state of PYK2 (Fig. 2d, e). These experiments show that bradykinin induces rapid tyrosine phosphorylation of PYK2 in PC12 cells. In contrast with stimulation of PYK2 phosphorylation in response to carbachol treatment (Fig. 2a) or membrane depolarization (Fig. 2b), the effect of bradykinin was not influenced by the omission of extracellular calcium (Fig. 2e): bradykinin induced PYK2 phosphorylation in the absence of extracellular calcium and in the presence of EGTA. PYK2 activation therefore appears to be a direct response to elevation of cytosolic calcium itself.

Incubation of PC12 cells with phorbol myristyl acetate (PMA) induced tyrosine phosphorylation of PYK2 (Fig. 2e), suggesting that tyrosine phosphorylation of PYK2 could also be mediated

**FIG. 2** Tyrosine phosphorylation of PYK2 in response to carbachol (carb.), bradykinin, membrane depolarization (m. depol.) and  $\text{Ca}^{2+}$  influx. **a**, Carbachol induces tyrosine phosphorylation of PYK2 by activation of the nicotinic acetylcholine receptor. Immunoprecipitates of PYK2 from PC12 cells that were subjected to the following treatments: muscarine (1 mM) or carbachol (carb.) (1 mM) for 20 s at 37 °C (left); carbachol (1 mM), DMPP (100  $\mu\text{M}$ ), or carbachol after pretreatment with the muscarinic antagonist atropine (100 nM) or the nicotinic antagonist mecamylamine (meca.) (10  $\mu\text{M}$ ) for 5 min at 37 °C (right). Incubation with carbachol in the presence or absence of EGTA (3 mM) as indicated (lower panels). The immunocomplexes were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with either anti-phosphotyrosine antibodies or with anti-PYK2 antibodies as indicated. **b**, Membrane depolarization and calcium ionophore induce tyrosine phosphorylation of PYK2. Immunoprecipitates of PYK2 from quiescent PC12 cells that were subjected to the following treatments: incubation with 75 mM KCl in the presence or absence of EGTA (3 mM) (left), incubation with 6  $\mu\text{M}$  of the calcium ionophore A23187 for 15 min at 37 °C (right). The immunoprecipitates were washed, resolved by 7.5% SDS-PAGE and immunoblotted with either anti-phosphotyrosine antibodies or with anti-PYK2 antibodies. **c**, Tyrosine phosphorylation of exogenous substrate by PYK2. PYK2 was immunoprecipitated from quiescent PC12 cells or following stimulation with carbachol or KCl in the absence or presence of EGTA. The immunoprecipitates were subjected to an *in vitro* kinase assay in the presence of [ $\gamma$ - $^{32}\text{P}$ ]ATP and the exogenous substrate poly(Glu-Tyr) (4:1). The phosphorylated products were resolved by SDS-PAGE, analysed by autoradiography (left) and quantified by a phospho-imager and Image Quant software (Molecular Dynamics, Inc.). The data are presented as kinase activity relative to unstimulated cells (right). **d**, Time course of bradykinin-induced tyrosine phosphorylation of PYK2. Quiescent PC12 cells were incubated at 37 °C with 1  $\mu\text{M}$  bradykinin (brad.) for the indicated periods of time. PYK2 was immunoprecipitated from untreated (–) or treated cells. The immunocomplexes were washed, resolved by SDS-PAGE, transferred to nitrocellulose, and probed either with anti-phosphotyrosine or anti-PYK2 antibodies (upper and lower panels, respectively). **e**, Quiescent PC12 cells were incubated with either 1  $\mu\text{M}$  bradykinin (1 min at 37 °C) or with PMA (1.6  $\mu\text{M}$ , 15 min at 37 °C) in the presence or absence of  $\text{CaCl}_2$  or EGTA (3 mM) as indicated. In some cases the cells were pretreated with 100 nM PMA for 12 h (indicated by the line above the lanes). PYK2 was immunoprecipitated from stimulated or unstimulated cells (–) and analysed by immunoblot analysis with either anti-phosphotyrosine or anti-PYK2 antibodies.

**METHODS.** Confluent PC12 cells in 150-mm plates were grown for 18 h in DMEM containing 0.5% horse serum and 0.25% fetal bovine serum. The cells were stimulated at 37 °C with different agonists as indicated, washed with cold PBS and lysed in 800  $\mu\text{l}$  lysis buffer<sup>47</sup>. The cell lysates were subjected to immunoprecipitation with anti-PYK2 antibodies. Following SDS-PAGE and transfer to nitrocellulose, the samples were immunoblotted with either anti-phosphotyrosine (RC20, Transduction Laboratories) or anti-PYK2 antibodies. Antibodies against PYK2 were raised in rabbits immunized with GST fusion protein containing amino acids 362–647 of PYK2 or against a synthetic peptide corresponding to 15 N-terminal residues. For exogenous substrate phosphorylation, PYK2 was immunoprecipitated with anti-peptide antibodies from serum-starved PC12 cells following stimulation for 3 min with carbachol (1 mM), KCl (75 mM) in the absence or presence of EGTA (3 mM) or left unstimulated, as indicated. The immunoprecipitates were washed twice with lysis buffer, once with Tris buffer (50 mM Tris-HCl, pH 7.5, 10 mM  $\text{MnCl}_2$ ) and incubated with 30  $\mu\text{l}$  Tris buffer containing 20  $\mu\text{g}$  poly(Glu-Tyr) (4:1) and 5  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]ATP (3,000 Ci mmol<sup>–1</sup>; NEN) for 12 min at 23 °C. The reactions were stopped by SDS sample buffer and resolved on 8.5% SDS-PAGE. Chronic treatment with PMA was performed by incubation of the cells with 100 nM PMA for 12 h at 37 °C.

by PKC activation. To test this hypothesis, PMA-sensitive isozymes were down-regulated by prolonged treatment with PMA<sup>20</sup>, and the cells were then treated with bradykinin or PMA. This prolonged treatment with PMA completely abolished the subsequent effect of PMA treatment (Fig. 2e), but had only a minor effect on bradykinin-stimulated tyrosine phosphorylation of PYK2. This suggests that tyrosine phosphorylation of PYK2 can be induced both by PKC-independent and PKC-dependent mechanisms, and that the effect of bradykinin is likely to be due to calcium release from intracellular stores. Chronic treatment



with phorbol ester also had a minor effect on carbachol-induced activation of PYK2 (data not shown), indicating that the effect of carbachol on PYK2 activation is also mediated by calcium influx, and not by PKC isozymes sensitive to phorbol ester.

We have demonstrated that PYK2 is rapidly activated in response to extracellular stimuli that increase intracellular calcium as a result of ion channel activation and membrane depolarization. PYK2 is also activated in response to stimulation of a G-protein-coupled receptor, as well as following stimulation by phorbol ester. Although the molecular mechanisms by which

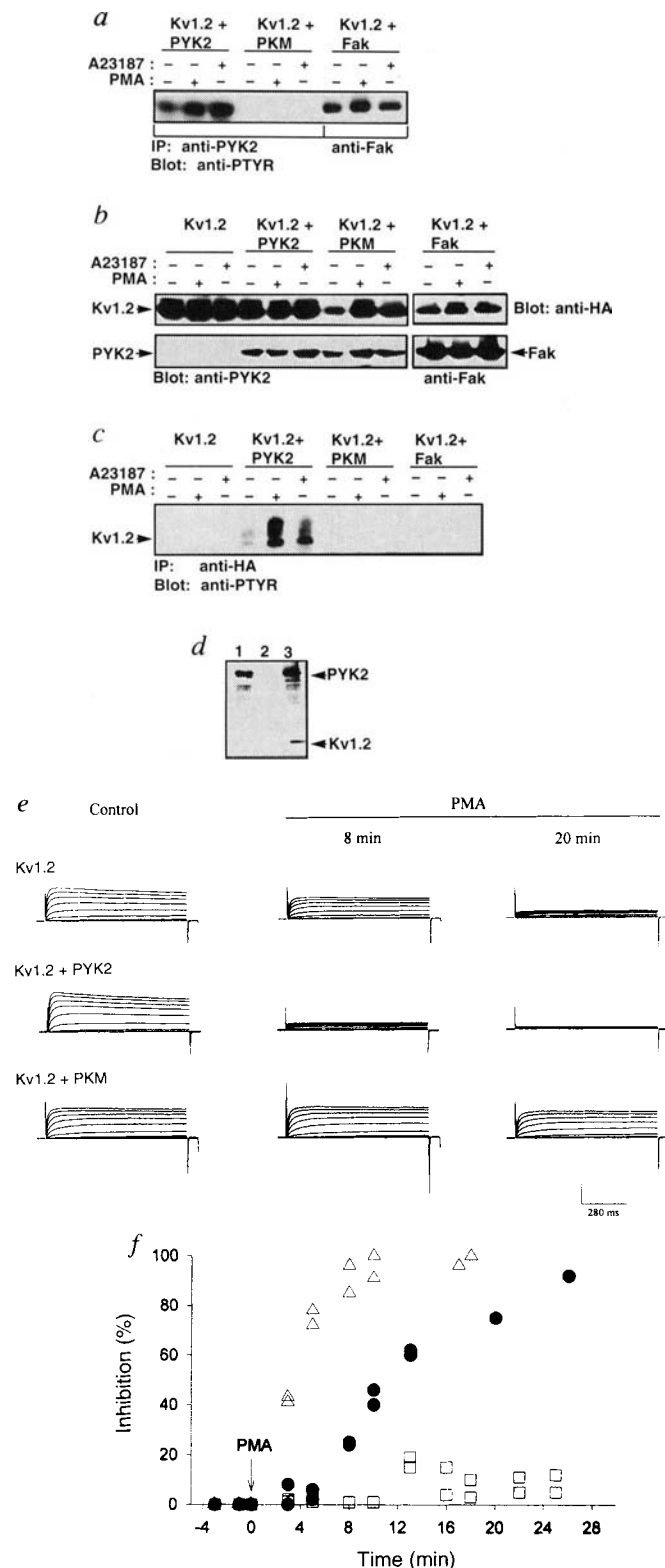


FIG. 3 Stimulation of Kv1.2 potassium channel tyrosine phosphorylation in response to PYK2 activation and suppression of Kv1.2 currents in *Xenopus* oocytes by PMA treatment and PYK2 expression. Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors which direct the synthesis of Kv1.2-HA, PYK2, a kinase-negative PYK2 (PKM) or the protein tyrosine kinase Fak. The cells were grown for 24 h in the presence of 0.2% serum and then either stimulated with PMA (1.6  $\mu$ M, 10 min at 37  $^{\circ}$ C), with the calcium ionophore A23187 (6  $\mu$ M, 10 min at 37  $^{\circ}$ C) or left unstimulated (–). a, Tyrosine phosphorylation of each protein was analysed following immunoprecipitation and immunoblotting with anti-

these signals induce the activation of PYK2 are not yet known, our results clearly show that elevation of intracellular calcium concentrations is crucial for PYK2 activation. We could not detect any significant effect of  $\text{Ca}^{2+}$  on the activity of isolated PYK2, indicating that  $\text{Ca}^{2+}$ -induced PYK2 activator is indirect. The effect of PMA on PYK2 activation indicates that PYK2 can also be activated by a PKC-dependent pathway.

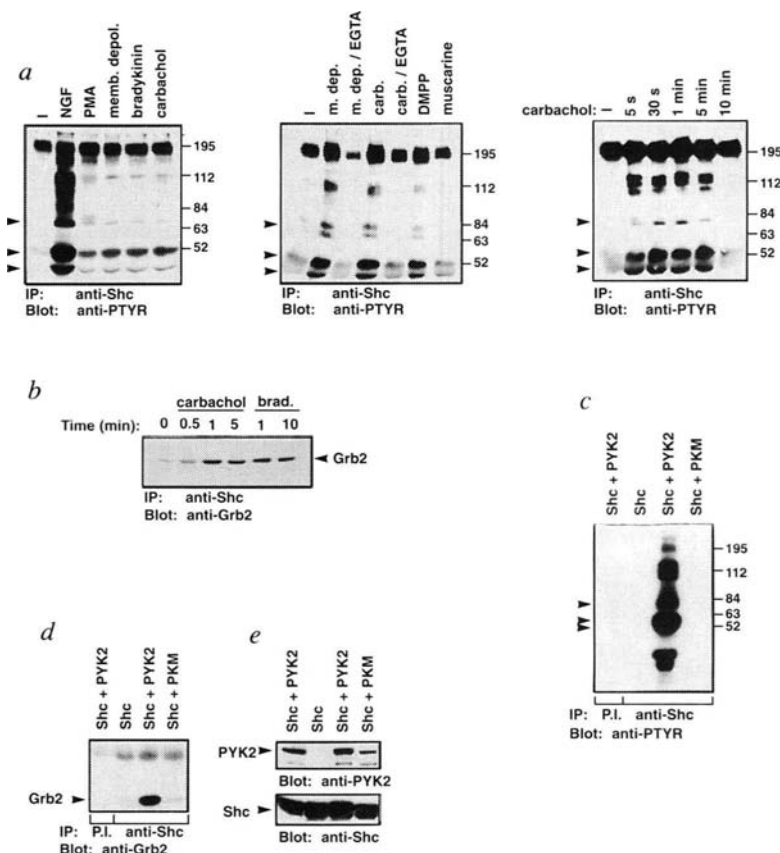
### Tyrosine phosphorylation of ion channels

That PYK2 can be activated by an ion channel, such as the nicotinic acetylcholine receptor, as well as by intracellular calcium, raised the possibility that PYK2 may in turn regulate ion channel function by tyrosine phosphorylation. Indeed, tyrosine phosphorylation can regulate the function of several ion channels in the central nervous system<sup>21-23</sup>. The delayed rectifier-type  $\text{K}^+$  channel, termed Kv1.2 (also called RAK, RBK2, RCK5, HukIV and NGK1)<sup>24</sup>, which is highly expressed in the brain and cardiac muscle<sup>25,26</sup>, can be regulated by tyrosine phosphorylation. It has been shown that tyrosine phosphorylation of Kv1.2 is associated with suppression of Kv1.2 currents, inhibition of which has been induced by a variety of stimuli, including carbachol, bradykinin, PMA and calcium ionophore<sup>12</sup>; note that the same stimuli are able to induce tyrosine phosphorylation of PYK2 (Fig. 2). We therefore examined whether PYK2 can phosphorylate the Kv1.2 channel on tyrosine and thus regulate its function. To test this possibility, we expressed in 293 cells the Kv1.2 protein, Kv1.2 together with PYK2, and, as a control, Kv1.2 with a kinase-negative PYK2 mutant (PKM) or the protein tyrosine kinase Fak. The cells were grown for 24 hours in medium containing 0.2% serum and were then stimulated with

PMA (1.6  $\mu\text{M}$ ), calcium ionophore (6  $\mu\text{M}$ ), or were left unstimulated. The expression level of these proteins in each cell line was determined by immunoblotting of total cell lysates with specific antibodies, as shown in Fig. 3b. Immunoblot analysis with anti-phosphotyrosine antibodies after immunoprecipitation of transiently expressed PYK2, PKM and Fak by specific antibodies is shown in Fig. 3a. PYK2 and Fak but not PKM were phosphorylated on tyrosine in unstimulated cells. Like many protein tyrosine kinases, PYK2 was found to be tyrosine phosphorylated on overexpression in 293 cells. Under these conditions both PMA and A23187 induced weak tyrosine phosphorylation of PYK2 (Fig. 3a). It is also possible that under these conditions the activity of PYK2 does not correlate well with phosphotyrosine content.

We next analysed the tyrosine phosphorylation of Kv1.2 channel in each cell line. We added a haemagglutinin (HA) tag to the cDNA expression construct of Kv1.2 and determined the level of Kv1.2 expression by immunoblot analysis with anti-HA antibodies. A similar amount of Kv1.2 protein was expressed in each of the transfected cell lines (Fig. 3b, top). Kv1.2 was immunoprecipitated from unstimulated cells, as well as from cells stimulated with PMA or calcium ionophore. The immunoprecipitates were resolved by SDS-PAGE and immunoblotted with anti-phosphotyrosine antibodies. Phosphorylation of Kv1.2 on tyrosine residues was observed only in cells coexpressing PYK2 (Fig. 3c). Tyrosine phosphorylation of Kv1.2 was enhanced by PMA or calcium ionophore treatments, indicating that activation of PYK2 is required for PYK2-induced tyrosine phosphorylation of the potassium channel. Moreover, immunoprecipitated PYK2 was able to tyrosine phosphorylate isolated

**FIG. 4** Tyrosine phosphorylation of Shc and its association with Grb2 in response to activation of PC12 cells and by overexpression of PYK2. **a**, Tyrosine phosphorylation of Shc in response to bradykinin, carbachol, PMA and other stimuli. Quiescent PC12 cells were stimulated for 5 min at 37 °C with bradykinin (1  $\mu\text{M}$ ), carbachol (1 mM), KCl (75 mM), PMA (1.6  $\mu\text{M}$ ), NGF (100 ng ml<sup>-1</sup>), or left unstimulated (–) (left). The cells were also stimulated with carbachol (1 mM) or potassium chloride (75 mM) in the presence of 3 mM EGTA as indicated. Stimulations with DMPP (100  $\mu\text{M}$ ) or muscarine (1 mM) were performed under the same conditions (middle). Time course of carbachol-induced tyrosine phosphorylation of Shc was performed by incubation of the cells with 1 mM carbachol for the indicated periods of time (right). The Shc proteins were immunoprecipitated with anti-Shc antibodies, the immunoprecipitates were resolved by SDS-PAGE (8%), transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine antibodies. **b**, Association between Grb2 and Shc in carbachol or bradykinin-stimulated PC12 cells. PC12 cells were treated with carbachol or bradykinin and then subjected to immunoprecipitation with anti-Shc followed by immunoblotting with Grb2 antibodies. **c**, Tyrosine phosphorylation of Shc in 293 cells that express Shc alone or coexpress Shc together with either PYK2, or PKM. The cells were lysed and subjected to immunoprecipitation with anti-Shc antibodies or preimmune serum (PI) as indicated. The immunocomplexes were washed, run on an SDS gel and immunoblotted with anti-phosphotyrosine antibodies. Shc proteins (of *M*<sub>r</sub> 46K, 52K and 66K) are marked by arrows. **d**, PYK2 induces association of Shc with Grb2. Shc proteins were immunoprecipitated from each cell line using anti-Shc antibodies. As a control, the lysates of cells that coexpress PYK2 and Shc were subject to immunoprecipitation with preimmune serum (P.I.). The presence of Grb2 in the immunocomplexes was determined by immunoblotting with anti-Grb2 antibodies. **e**, The expression level of PYK2, PKM (top) and Shc (bottom) in each cell line was determined by immunoblot analysis of total cell lysates with specific antibodies as indicated. **METHODS**. PC12 cells were grown in DMEM containing 0.25% fetal bovine serum and 0.5% horse serum for 18 h before stimulation. After stimulation, the cells were washed with cold PBS and lysed in 0.8 ml



lysis buffer<sup>47</sup>. PYK2 was immunoprecipitated by anti-PYK2 antibodies, the immunoprecipitates were resolved by 7.5% SDS-PAGE and immunoblotted either with anti-phosphotyrosine antibodies (RC20, Transduction Laboratories, Lexington, Kentucky) or with anti-PYK2 antibodies.



Kv1.2 channel protein (Fig. 3d), suggesting that tyrosine phosphorylation of the channel by PYK2 is direct.

### Suppression of channel currents

Because tyrosine phosphorylation of Kv1.2 is known to be associated with the suppression of Kv1.2 currents<sup>12</sup>, and PYK2 can induce tyrosine phosphorylation of Kv1.2 (Fig. 3c), we investigated whether stimulation of PYK2 can suppress Kv1.2 currents. We explored the effect of PYK2 expression on currents generated by Kv1.2 expression in *Xenopus* oocytes. Stage V oocytes were microinjected either with Kv1.2 mRNA alone or with Kv1.2 together with PYK2 or PKM mRNAs. After 2–3 days of incubation at 20 °C, macroscopic currents exhibited by the oocytes were recorded with a two-microelectrode voltage clamp as previously described<sup>27</sup>. Large outward rectifier currents were recorded upon membrane depolarization above –40 mV, indicating that functional Kv1.2 channels are expressed in the oocytes. The expression of Kv1.2, PYK2 and PKM in the frog oocytes was confirmed by immunoblot analysis with anti-HA or anti-PYK2 antibodies (data not shown).

It has previously been shown that Kv1.2 currents in *Xenopus* oocytes can be inhibited in response to PMA treatment or elevation of intracellular calcium concentration<sup>12</sup>. This suppression is mediated by an unknown endogenous protein tyrosine kinase(s) that can phosphorylate the Kv1.2 channels<sup>12</sup>. Because PYK2 can be activated by PMA, we examined the effect of PYK2 expression on Kv1.2 currents in oocytes in the absence or presence of PMA. We also examined the effect of the kinase-negative mutant PKM on PMA-induced suppression of Kv1.2 currents mediated by the endogenous protein tyrosine kinase. PMA treatment of oocytes that were injected with Kv1.2 mRNA were found to cause inhibition of Kv1.2 currents (Fig. 3e). As previously

shown, the inhibition of the currents developed gradually after application of PMA, reaching 80–90% inhibition after approximately 20 min incubation<sup>12</sup>. Moreover, the rate of channel blockade was found to be dependent on the concentration of PMA applied (data not shown). Coexpression of PYK2 resulted in an acceleration of Kv1.2 current inhibition (Fig. 3e,f), which was observed at every concentration of PMA tested. For example, 8 min after the addition of 100 nM PMA, 25% inhibition of outward current was observed in oocytes expressing Kv1.2 alone, as compared with 85% inhibition observed in oocytes coexpressing Kv1.2 and PYK2 (Fig. 3e,f). Current inhibition by PMA treatment in the absence or presence of PYK2 expression did not result in changes in both the kinetics or voltage dependence of the remaining currents (Fig. 3e). Interestingly, coexpression of Kv1.2 and PKM led to nearly complete inhibition of PMA-induced potassium channel blockage (Fig. 3e,f). It is possible that the endogenous protein tyrosine kinase activated by PMA responsible for suppression of Kv1.2 currents in oocytes represents the *Xenopus* homologue of PYK2 or a closely related protein tyrosine kinase that can be affected by a dominant interfering mutant of PYK2.

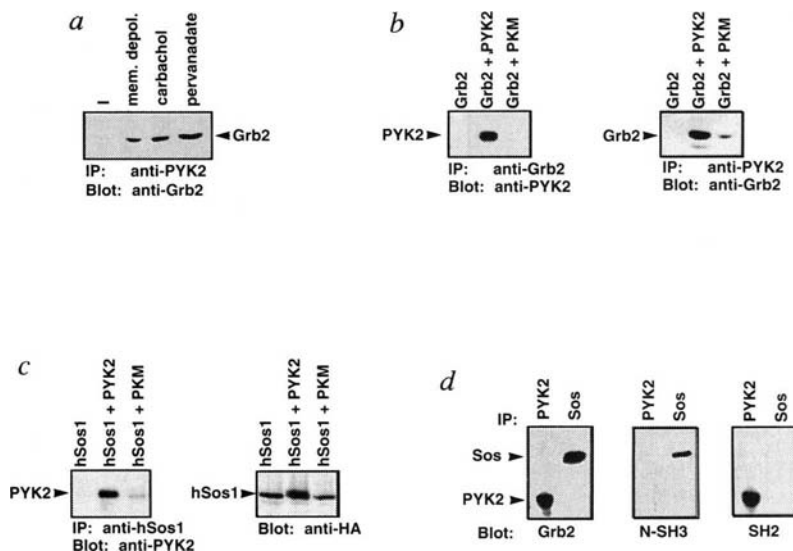
### Recruitment of Shc/Grb2/Sos complexes

The results presented so far suggest that PYK2 activation provides an element that translates increases in intracellular calcium induced by neuronal activity into functional changes. It has been shown that calcium influx by voltage-sensitive calcium channels leads to MAP kinase activation in PC12 cells<sup>28</sup>. We therefore analysed the role of PYK2 in MAP kinase activation.

The stimuli that induce the activation of PYK2 also induce tyrosine phosphorylation of the adaptor protein Shc. Treatment of PC12 cells with bradykinin, carbachol, 75 mM potassium

**FIG. 5 Association of PYK2 with Grb 2 and Sos1 in intact cells and *in vitro*.** *a*, Association between Grb2 and PYK2 in stimulated PC12 cells. PC12 cells were stimulated with KCl, carbachol and pervanadate and subjected to immunoprecipitation with anti-PYK2 antibodies followed by immunoblotting with anti-Grb2 antibodies. *b*, Embryonic human kidney 293 cells were transiently transfected with different combinations of mammalian expression vectors that direct the synthesis of Grb2, PYK2 and a kinase-negative PYK2 mutant (PKM). The cells were made soluble and immunoprecipitated with anti-Grb2 (left) or anti-PYK2 antibodies (right). The immunocomplexes were washed, resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with either anti-PYK2 (left) or anti-Grb2 antibodies (right). *c*, Embryonic human kidney 293 cells were transiently transfected with mammalian expression vectors encoding hSos1-HA, hSos1-HA together with PYK2, or hSos1-HA together with PKM. hSos1 was immunoprecipitated with anti-hSos1 antibodies from each cell line, and the presence of PYK2 in the immunocomplexes was determined by immunoblotting with anti-PYK2 antibodies (left). Expression levels of hSos1 (right), was determined by immunoblot analysis of total cell lysates with anti-HA antibodies. *d*, Direct binding of Grb2 SH2 domain to PYK2. Human embryonic kidney 293 cells were transfected with expression vectors that direct the synthesis of PYK2 or hSos1. These proteins were immunoprecipitated as indicated, resolved by SDS-PAGE and transferred to nitrocellulose filters. The filters were incubated with GST-fusion proteins of Grb2, Grb2-SH2 domain as well as Grb2-N-SH3 and C-SH3 domains. The binding of the fusion proteins to the filters was detected by anti-GST antibodies followed by protein A conjugated to horseradish peroxidase. The binding of Grb2, Grb2-SH2 domain and Grb2-N-SH3 domain to PYK2 and Sos1 are shown. C-SH3 domain does not bind to PYK2 or to Sos1.

**METHODS.** Quiescent PC12 cells were stimulated with carbachol (1  $\mu$ M), KCl (75 mM) or pervanadate (100  $\mu$ M) for 4 min at 37 °C or left unstimulated. PYK2 was immunoprecipitated with a mixture of anti-PYK2 and anti-peptide antibodies (see Fig. 2) and the presence of Grb2

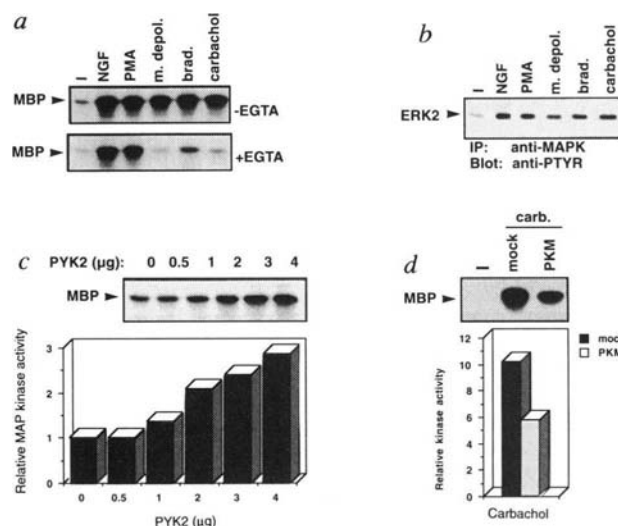


in the immunocomplex was indicated by immunoblotting with anti-Grb2 antibodies (Transduction Laboratories). 293 cells were transiently transfected with the full-length cDNAs of PYK2, PKM Grb2 and hSos1-HA cloned into the mammalian expression vector pCMP1 downstream of the CMV promoter, using the calcium phosphate precipitation method<sup>48</sup>. Full details about the antibodies and protocols used for the immunoprecipitation of Grb2 and Sos have been described previously<sup>50</sup>. A mammalian expression vector that encodes the hSos1-HA was constructed as described<sup>50</sup>. The recombinant fusion proteins GST-Grb2, GST-SH2, GST-N-SH3 and GST-C-SH3 (5  $\mu$ g ml<sup>-1</sup>) were incubated with PYK2 or Sos1 immobilized on nitrocellulose filters for 1 h at room temperature. Following extensive washing, filters were incubated with rabbit polyclonal antibodies against GST and probed with protein A conjugated to horseradish peroxidase.

chloride (to induce membrane depolarization), PMA and, as a control, nerve growth factor (NGF) resulted in tyrosine phosphorylation of Shc as well as several associated proteins (Fig. 4a). Tyrosine phosphorylation of Shc in response to membrane depolarization and carbachol treatment was dependent on the presence of extracellular calcium (Fig. 4a, middle), indicating that calcium influx is involved in the regulation of Shc phosphorylation by these stimuli. Tyrosine phosphorylation of Shc in response to carbachol treatment is induced by means of stimulation of the nicotinic acetylcholine receptor, as determined by pharmacological analysis (Fig. 4a, middle panel). The effect of carbachol on tyrosine phosphorylation of Shc was transient with maximum tyrosine phosphorylation detected after one minute, followed by a rapid decline (Fig. 4a, right). However, NGF induced persistent stimulation of Shc phosphorylation for at least 5 hours after factor addition<sup>29</sup>. The duration of Shc phosphorylation may have an important impact on the Ras signalling pathway and gene expression induced by these stimuli<sup>30,31</sup>.

The activated EGF receptor can recruit Grb2 directly, and also indirectly by tyrosine phosphorylation of Shc<sup>32–34</sup>. We have therefore investigated whether PYK2 can induce tyrosine phosphorylation of Shc, and explored its association with Grb2 in PC12 cells stimulated with carbachol or bradykinin. These treatments induced complex formation between Grb2 and Shc only in stimulated PC12 cells (Fig. 4b). Similar results were obtained in 293 cells expressing PYK2 (Fig. 4). In this experiment, Shc proteins were immunoprecipitated with anti-Shc antibodies from Shc, from Shc and PYK2, or from Shc and PKM-expressing cells. The samples were resolved by SDS-PAGE, transferred to nitrocellulose and immunoblotted with anti-phosphotyrosine (Fig. 4c) or anti-Grb2 antibodies (Fig. 4d). This experiment reveals dramatic tyrosine phosphorylation of Shc in cells that overexpress PYK2. Moreover, several phosphotyrosine-containing proteins were found in Shc immunoprecipitates from cells overexpressing PYK2. Similar results were also obtained in cells expressing endogenous Shc proteins that were transfected with PYK2 cDNA and subjected to immunoprecipitation analysis with anti-Shc antibodies (data not shown). Immunoblot analysis with Grb2 antibodies of Shc immunoprecipitates (Fig. 4d) indicated that Grb2 associates with tyrosine-phosphorylated Shc in cells overexpressing PYK2. However, direct association between PYK2 and Shc was not detected.

Shc was shown to be involved in the coupling of both receptor and non-receptor tyrosine kinases to the Ras/MAPK signalling pathway<sup>33,35</sup>. Tyrosine-phosphorylated Shc can activate the Ras signalling pathway by binding to the SH2 domain of the adaptor protein Grb2 that is complexed to the guanine-nucleotide-releasing factor Sos by means of its SH3 domains<sup>32,33</sup>. The MAP kinase signalling pathway in PC12 cells can be activated by NGF<sup>36</sup>, by peptide hormones that activate G-protein-coupled receptors<sup>37</sup>, by phorbol ester<sup>38</sup>, and by calcium influx following membrane depolarization<sup>28</sup>. However, the mechanism underlying activation of the Ras/MAP kinase signalling pathway by G-protein-coupled receptors and by calcium influx are not known. To explore the possibility that calcium-induced PYK2 activation is responsible for activation of the Ras/MAPK signalling pathway, we examined the interaction between the adaptor protein Grb2 with PYK2 in PC12 cells stimulated by carbachol or KCl. Carbachol or KCl treatments led to complex formation between Grb2 and PYK2 in stimulated PC12 cells (Fig. 5a). Similar results were obtained in 293 cells transfected with different combinations of expression vectors that direct the synthesis of PYK2, PKM and the adaptor protein Grb2. Grb2 is directly associated with wild-type PYK2 but not with PKM (Fig. 5b). Experiments with glutathione *S*-transferase (GST) fusion proteins indicate that the association is mediated by the SH2 domain of Grb2 (Fig. 5d). Inspection of the PYK2 primary structure shows that Tyr 881 is followed by an LNV sequence that was shown to be a canonical binding site for the SH2 domain of Grb2 (ref. 33).



**FIG. 6** Activation of MAP kinase in PC12 cells by different agonists. Quiescent PC12 cells were stimulated for 5 min at 37 °C with bradykinin (1 µM), carbachol (1 mM), KCl (75 mM), PMA (1.6 µM), NGF (100 ng ml<sup>-1</sup>) or left unstimulated (–) in the absence or presence of EGTA (3 mM). The cells were lysed and ERK1,2 were immunoprecipitated with specific antibodies. The immunocomplexes were washed and subjected to a standard MBP phosphorylation assay (a) or resolved by SDS-PAGE, transferred to nitrocellulose filters and immunoblotted with anti-phosphotyrosine antibodies (b). c, Activation of MAP kinase by overexpression of PYK2. Human embryonic kidney 293 cells were transfected with increasing concentrations of a mammalian expression vector that directs the synthesis of PYK2. ERK2 protein was immunoprecipitated from each cell line, and the immunocomplexes were washed and subjected to MBP phosphorylation assay (top). Quantification of MAP kinase activity for each cell line was determined by phosphorimager and Image Quant software (Molecular Dynamics). MAP kinase activity in transfected cells is compared to activity detected in control mock-transfected cells (bottom). d, Suppression of carbachol-induced MAP kinase activation by overexpression of kinase-negative mutant PKM in PC12 cells. Stimulation of PC12 with carbachol, immunoprecipitation and MBP phosphorylation are described in a. MBP phosphorylation (top); quantification of MAP kinase activity (bottom). **METHODS.** PC12 cells were starved for 18 h as described. The cells were stimulated for 5 min at 37 °C with the indicated stimuli, lysed and subjected to immunoprecipitation with anti-ERK 1 or 2 antibodies as indicated (Santa Cruz Biotechnology, SC-93 or SC 154). The immunoprecipitates were washed twice with lysis buffer<sup>47</sup>, and once with Tris buffer containing 10 mM Tris-HCl, pH 7.2, 100 mM NaCl, 1 mM Na-vanadate and 5-mM benzamide. The immunocomplexes were resuspended in 40 µl MAP kinase buffer containing 30 mM Tris-HCl, pH 8, 20 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, 15 µg MBP, 10 µM ATP and 5 µCi [γ-<sup>32</sup>P]ATP (Amersham). The samples were incubated for 3 min at 30 °C and the reactions were stopped by the addition of SDS-sample buffer. The samples were resolved on 15% SDS-PAGE and analysed by autoradiography. Human embryonic kidney 293 cells were transiently transfected with increasing concentrations of pCMP1-PYK2 DNA (0.5–4 µg). Then, 12 h after transfection, the cells were grown in medium containing 0.2% serum for 24 h. The cells were lysed, immunoprecipitated with ERK2 antibodies and subjected to MBP phosphorylation assay as described above. PC12 cells in 65-mm plates were transiently transfected with 10 µg DNA of pCMP1 or pCMP1-pKM using lipofectamine reagents (GIBCO-BRL). The cells were starved for 24 h, stimulated with 1 mM carbachol for 5 min and subjected to MAP kinase activity assay as described.

This site is equivalent to Tyr 925 of Fak, a known Grb2 binding site<sup>39</sup>.

We next examined the interaction of PYK2 with the guanine nucleotide releasing factor Sos1. Human embryonic kidney 293 cells were transfected with expression vectors encoding Sos1 together with either PYK2 or PKM, and subjected to immunoprecipitation/immunoblotting analysis with anti-Sos1 or anti-

PYK2 antibodies, respectively. Wild-type PYK2, but not the kinase-negative mutant PKM, was co-immunoprecipitated with the Sos1 protein. Hence Grb2 is bound to Sos1 by means of its SH3 domains, and to PYK2 via its SH2 domain (Fig. 5d), leading to the recruitment of Sos by tyrosine-phosphorylated PYK2. Growth-factor-induced activation of receptor tyrosine kinases leads to a shift in the electrophoretic mobility of Sos protein. The mobility shift is due to phosphorylation by serine and threonine kinases that are dependent on Ras activation, including the MAP kinase<sup>33,40</sup>. Sos1 protein from PYK2 transfected cells exhibits reduced electrophoretic mobility compared to Sos1 protein from PKM-expressing cells (Fig. 5c, right). This shows that PYK2 overexpression leads to the activation of Ser/Thr kinases that are responsible for the phosphorylation of Sos1. We therefore concluded that tyrosine-phosphorylated PYK2 can directly and indirectly recruit Grb2 by means of tyrosine phosphorylation of Shc, revealing at least two alternative routes for PYK2-induced activation of the Ras signalling pathway.

We next examined the ability of these stimuli to induce the activation of MAP kinase in PC12 cells. Quiescent PC12 cells were stimulated with different agonists in the absence or presence of EGTA (Fig. 6a). Lysates from stimulated cells were immunoprecipitated with anti-MAP kinase (ERK1,2) antibodies, and MAP kinase activity was analysed by using myelin basic protein (MBP) as an exogenous substrate (Fig. 6a). In parallel, ERK2 immunoprecipitates were transferred to nitrocellulose filters and probed with phosphotyrosine antibodies (Fig. 6b). This demonstrated that the addition of various agonists to PC12 cells induced a similar profile of tyrosine phosphorylation and MAP kinase activation. It is clear that MAP kinase activation is induced by both calcium-influx-dependent and independent mechanisms.

Because activation of MAP kinase was observed in response to stimuli that induce PYK2 phosphorylation, we examined the possibility that PYK2 overexpression could induce MAP kinase activation. Human embryonic kidney 293 cells were transiently transfected with increasing concentrations of mammalian expression vector that directs the synthesis of PYK2. The cells were grown for 24 hours in the presence of 0.2% serum, and ERK2 proteins were immunoprecipitated, washed and subjected to MBP phosphorylation assay. PYK2 overexpression induced MBP phosphorylation in a concentration-dependent manner (Fig. 6c). However, overexpression of the kinase negative mutant PKM suppressed carbachol-induced activation of MAP kinase (Fig. 6d), probably by dominant negative suppression of agonist-induced activation of endogenous PYK2 in PC12 cells.

## Discussion

We have discovered a protein tyrosine kinase termed PYK2 that is highly expressed in the adult rat brain. PYK2 is a second member of the Fak family of non-receptor protein tyrosine kinases. We present data demonstrating that PYK2 is activated in response to bradykinin, a neuropeptide hormone that mediates its biological effects by activation of a G-protein-coupled receptor and by stimulation of phosphatidylinositol hydrolysis. PYK2 is also tyrosine-phosphorylated in response to activation of the nicotinic acetylcholine receptor, as well as by membrane depolarization and calcium-ionophore treatment. All of these stimuli result in enhanced levels of cytosolic calcium influx. PYK2 is thus activated by extracellular signals that lead to calcium influx or calcium release from internal stores. Moreover, PYK2 appears to be regulated by both PKC-dependent and independent mechanisms. Our experiments show that PYK2 may also link G-protein-coupled receptors and calcium influx to the MAP kinase signalling pathway, which relays signals from the cell surface to regulate transcriptional events in the nucleus. Moreover, the effects of PYK2 on tyrosine phosphorylation and action of the Kv1.2 potassium channel provide a mechanism for heterologous regulation of ion channel function by activation of an intermediate protein tyrosine kinase. PYK2 can, therefore,

link neuropeptide hormones that act by means of G-protein-coupled receptors, stimulating phosphatidylinositol hydrolysis with the action of target channel molecules. Similarly, PYK2 may modulate the action of ion channels, such as voltage-gated calcium channels, that mediate their responses by, and are sensitive to, intracellular calcium concentration. PYK2 may therefore provide an autoregulatory role for the same channel that is responsible for PYK2 activation. One potential target of PYK2 is the nicotinic acetylcholine receptor.

Calcium concentration inside cells is highly localized because of a variety of calcium-binding proteins that provide a strong buffer<sup>41</sup>. Moreover, in excitable cells the level of calcium can be regulated by voltage-dependent calcium channels that induce a large and transient increase in intracellular calcium concentration, leading to calcium oscillations and calcium waves<sup>41</sup>. Because PYK2 activity is regulated by intracellular calcium level, both the temporal and spatial pattern of PYK2 activation may replicate the spatial and temporal profile of intracellular calcium concentration. An intriguing possibility, therefore, is that PYK2 and other calcium-dependent tyrosine kinases<sup>42</sup> may provide a mechanism for rapid and highly localized control of ion channel function, as well as localized activation of the MAP kinase signalling pathway. The effect of PMA on PYK2 activation also suggests a tight association between PKC and PYK2 activation.

Potassium channels are frequent targets for phosphorylation by protein kinases that are activated by neurotransmitters or neuropeptides<sup>43</sup>. Phosphorylation of these and other voltage-gated channels or neurotransmitter receptors provides an important regulatory mechanism for modulation of neuronal activity<sup>5</sup>. *In situ* hybridization and preliminary immunolocalization analyses indicate that PYK2 is expressed in hippocampal pyramidal cell dendrites (unpublished results), suggesting a role for this kinase in synaptic plasticity mediated by calcium influx after postsynaptic depolarization. In summary, PYK2 may represent an important signalling intermediate molecule between neuropeptides that activate G-protein-coupled receptors, or neurotransmitters that increase  $Ca^{2+}$  influx, and downstream signalling events that regulate neuronal activity. Moreover, our results suggest a mechanism for the coupling between various signals that elevate intracellular calcium and the MAP kinase signalling pathway: activation of MAP kinase leads to translocation of the enzyme to the nucleus to phosphorylate TCF/Elk-1, resulting in induction of *c-fos* transcription<sup>44</sup> and activation of AP-1 transcriptional factor<sup>45</sup>. PYK2 may also be involved in calcium-mediated regulation of gene expression in neuronal cells induced by NMDA receptor or voltage-sensitive calcium channels<sup>46</sup>. PYK2 therefore may control a broad array of processes in the central nervous system, including short- and long-term neuronal plasticity. □

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# Three-dimensional structure of the catalytic subunit of protein serine/threonine phosphatase-1

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**The crystal structure of mammalian protein phosphatase-1, complexed with the toxin microcystin and determined at 2.1 Å resolution, reveals that it is a metalloenzyme unrelated in architecture to the tyrosine phosphatases. Two metal ions are positioned by a central  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  scaffold at the active site, from which emanate three surface grooves that are potential binding sites for substrates and inhibitors. The carboxy terminus is positioned at the end of one of the grooves such that regulatory sequences following the domain might modulate function. The fold of the catalytic domain is expected to be closely preserved in protein phosphatases 2A and 2B (calcineurin).**

PROTEIN phosphatases were first discovered about fifty years ago as a cellular activity that converts glycogen phosphorylase from the active *a* form to the inactive *b* form (for a review, see ref. 1). The critical role that reversible phosphorylation is now known to play in cellular signal transduction places the protein phosphatases in a position of central importance because of their ability to reverse the action of protein kinases. Phosphatases that act on phosphorylated serine and threonine residues are present in all eukaryotic cell types and participate in the control of a wide range of cellular processes, including cell-cycle progression, cell proliferation, protein synthesis, transcriptional regulation and neurotransmission<sup>2,3</sup>. In contrast to the protein kinases, for which several three-dimensional structures have been elucidated, the serine/threonine phosphatases have as yet eluded crystallographic analysis and are less well understood in terms of structure and mechanism.

The serine/threonine phosphatases are unrelated in sequence to the protein tyrosine phosphatases, and have been classified into four groups (1, 2A, 2B, 2C) on the basis of the differences in their biochemical properties<sup>4</sup>. Protein phosphatases (PP) 1, 2A and 2B (also known as calcineurin) have highly homologous catalytic domains (Fig. 1), but differ in their substrate specificities and interactions with regulatory molecules. PP-2C is unrelated to these enzymes, and we shall here use the term Ser/Thr phosphatase to refer specifically to relatives of PP-1. Included with the Ser/Thr phosphatases is an enzyme encoded by

bacteriophage  $\lambda$  ( $\lambda$  phosphatase), which is homologous in sequence to the N-terminal half of PP-1 (Fig. 1) and has been a useful model for mechanistic studies<sup>5</sup>.

Ser/Thr phosphatases are subject to complex regulation. Proteins known as 'targeting subunits' form heterodimers with the catalytic subunit of PP-1, resulting in its specific localization and modulation of its inhibition by other factors<sup>6</sup>. The heat-stable inhibitors, inhibitor-1 and its homologue DARPP-32 (dopamine- and cyclic AMP-regulated phosphoprotein of apparent *M<sub>r</sub>* 32K), when phosphorylated by cAMP-dependent protein kinase, bind to PP-1 and inhibit it. DARPP-32 is highly enriched in certain types of neurons, where its phosphorylation links the dopamine activation of receptors to the control of Na<sup>+</sup>,K<sup>+</sup>-ATPase and the membrane potential<sup>7</sup>. PP-1 is also inhibited by inhibitor-2 (ref. 2) and by phosphorylation in a C-terminal region by a cyclin-dependent protein kinase<sup>8,9</sup>. Calcineurin is a heterodimer formed by a catalytic A subunit and a calmodulin-like B subunit<sup>10</sup>, and is activated by the binding of Ca<sup>2+</sup>-calmodulin to a regulatory region that follows the catalytic domain<sup>11</sup>.

Certain natural toxins of agricultural and medical importance are potent inhibitors of the Ser/Thr phosphatases<sup>12</sup>. PP-1 and PP-2A are inhibited by the membrane-permeable polyether okadaic acid and by cyclic heptapeptides known as microcystins. Both these toxins are powerful inducers of tumours<sup>13,14</sup>, which suggests a role for PP-1 and PP-2A in growth suppression. The immunosuppressants cyclosporin and FK506 inhibit calcineurin when bound to the proteins cyclophilin and FKBP, respectively, and the discovery of this phenomenon has revealed that calcineurin has a key role in T-cell signalling<sup>15</sup>.

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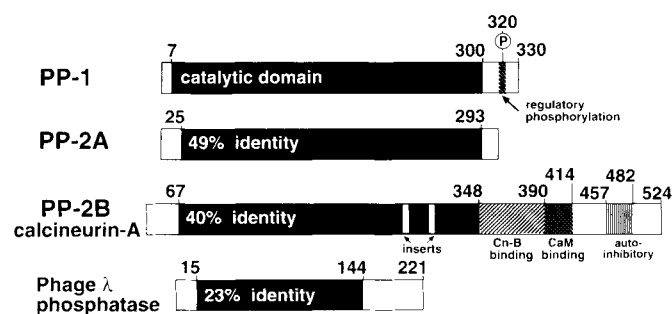


FIG. 1 Schematic diagram showing the domain structure and regulatory regions of Ser/Thr phosphatases. The residue numbers given refer to PP-1 from rabbit muscle<sup>36</sup>, human PP-2A<sup>37</sup>, human PP-2B (calcineurin-A)<sup>38</sup> and the phosphatase encoded by bacteriophage  $\lambda$ <sup>39</sup>. The black box in PP-1 denotes the ordered domain seen in the crystal structure. Homologous regions in the other proteins are also shown in black. The percentage sequence identity to PP-1 within these regions is shown for each protein. The site of inhibitory phosphorylation in PP-1 is indicated<sup>8,9</sup>. Residues in calcineurin-A implicated in the binding of calcineurin-B (CnB)<sup>40</sup> and  $\text{Ca}^{2+}$ /calmodulin (CaM)<sup>41</sup> are indicated by shading, as is an autoinhibitory region<sup>41</sup>.

**METHODS.** Full-length PP-1 ( $\alpha$ -isoform) rabbit muscle cDNA was expressed in *Escherichia coli* (provided by N. Berndt) and purified using heparin-Sepharose, phenyl-Sepharose, Sephacryl-S200 and MonoQ chromatography (Pharmacia) in the presence of 1 mM  $\text{MnCl}_2$ , except for the final MonoQ step, which was done in the absence of Mn. Protein was concentrated to 8 mg ml<sup>-1</sup> in 0.5 M NaCl and reacted with a 4-fold molar excess of microcystin-LR (Calbiochem). Orthorhombic crystals (space group,  $P2_12_12_1$ ,  $a = 64.8$  Å,  $b = 77.1$  Å,  $c = 130.7$  Å, two molecules in the asymmetric unit) were obtained by hanging-drop vapour diffusion by mixing 1  $\mu$ l of protein-microcystin complex in 50 mM Tris (pH 7.0), 500 mM NaCl, 10 mM  $\beta$ -mercaptoethanol, 0.9 mM  $\text{MnCl}_2$ , 0.1 mM EGTA with 0.8  $\mu$ l reservoir solution containing 65 mM Tris (pH 8.8), 200 mM  $\text{MgCl}_2$  and 32% polyethylene glycol (average  $M_r \sim 4,000$ ). An additional 0.2  $\mu$ l of 1.0 M 1,7-diaminoheptane (Fluka) improved the crystal morphology. An independent crystallization of the  $\gamma$ -isoform of PP-1 in a different space group has been reported recently<sup>42</sup>.

The three-dimensional structure of Ser/Thr phosphatases has not yet been reported, leaving many fundamental questions regarding their regulation and catalytic mechanism unanswered. We now describe the crystal structure of PP-1 complexed with the toxin microcystin, determined by X-ray crystallography at 2.1 Å resolution. The precise description of the protein architecture that results, coupled with the discovery of a two-metal coordination centre at the active site, now makes possible a structure-based dissection of the functions of this important class of regulatory proteins.

### Crystallographic analysis

Crystals of the PP-1/microcystin complex diffract X-rays strongly when frozen to  $\sim 100$  K, with reflections observed beyond 1.8-Å Bragg spacings. Determination of phases by multiple isomorphous replacement (MIR) was problematic because of non-isomorphism between native and derivative crystals. Finally, two mercury derivatives yielded phases of moderate quality, but the resulting electron density map was generally uninterpretable. One of the mercury derivatives was used to obtain multiwavelength anomalous diffraction (MAD) data at a synchrotron<sup>16</sup>, providing independent phase information that was combined with the MIR information to yield an interpretable electron density map (Table 1). The current atomic model has been refined against data to 2.1 Å resolution, with a crystallographic  $R$ -value of 17.8% (including all data).

### General features of the structure

Residues 7 to 300 of PP-1 form a compact ellipsoidal structure, referred to as the catalytic domain, with dimensions of

$\sim 50$  Å  $\times$   $35$  Å  $\times$   $35$  Å (Fig. 3). Comparison of the sequences of PP-1, PP-2A and calcineurin indicates that the conserved part of the domain starts at about residue 30 of PP-1, and continues until about residue 295 (refs 17, 18). To emphasize this, we label the  $\alpha$ -helices  $\alpha A$ ,  $\alpha B$ ,  $\alpha C$ , and the  $\beta$ -strands  $\beta 1$ ,  $\beta 2$ ,  $\beta 3$ , and so on, beginning with the second of the helices and strands (Fig. 3a). The first helix and strand ( $\alpha A'$ ,  $\beta 1'$ ) cap the hydrophobic core of the domain by packing against helices  $\alpha A$ ,  $\alpha B$  and  $\alpha C$  (Fig. 3b). These in turn pack against helices  $\alpha D$ ,  $\alpha E$  and  $\alpha F$ , and the cluster of these seven helices forms the base of the molecule. Helices  $\alpha B$  and  $\alpha C$  lie underneath a 5-stranded  $\beta$  sheet (sheet 1), formed by three parallel strands ( $\beta 2$ ,  $\beta 3$  and  $\beta 4$ ) and two antiparallel strands ( $\beta 13$  and  $\beta 14$ ) from the C-terminal end of the domain. Sheet 1 packs against another sheet (sheet 2) composed of strands  $\beta 1$ ,  $\beta 5$ ,  $\beta 6$ ,  $\beta 10$ ,  $\beta 11$  and  $\beta 12$ . Finally, an irregular arrangement of three helices ( $\alpha G$ ,  $\alpha H$ ,  $\alpha I$ ) and a 3-stranded  $\beta$  sheet ( $\beta 7$ ,  $\beta 8$ ,  $\beta 9$ ; sheet 3) caps the other side of sheet 2 and completes the structure. Two predictions of the secondary<sup>17,18</sup> and super-secondary<sup>18</sup> structure of Ser/Thr phosphatases have been published recently. Many, but not all, of the secondary structural features of PP-1 are predicted correctly. In both studies, the assignment of active-site regions was mostly correct and a role for predicted active-site residues in binding catalytic metal ions was suggested.

The catalytic domain can be divided conceptually into two tightly linked sub-structures. The N-terminal region (the 'N-subdomain', residues 7 to 182; coloured violet, blue, red and yellow in Fig. 3) includes a  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  metal-coordinating unit (red) and forms a compact structure in which two metal ions are embedded (Fig. 3b). The C-terminal region (the 'C-subdomain', residues 183-300; coloured green) forms an irregular structure that sits on top of the N-subdomain and provides potential binding sites for substrates and inhibitors (see later). This division is suggested by sequence comparison, which indicates that the N-subdomain is conserved in the  $\lambda$ -phosphatase, but that the C-terminal region of that enzyme may be unrelated (Fig. 1).

### Metal ions at the active site

The crystallographic data demonstrate unequivocally that PP-1 contains two metal ions that are close to each other, near the C termini of the  $\beta$ -strands of the  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  unit (Fig. 3a). Based on the location of the metals at the surface of the enzyme, as well as the strict conservation of the metal coordinating residues and the severe effect on catalysis caused by mutation of these residues<sup>5</sup> (discussed below), we conclude that the metals are located at the active site of the enzyme. The role of the metals in PP-1 does not appear to be primarily structural: the metal-binding site is constructed from regions of the protein core that appear to present the metals at the surface in a manner suitable for interaction with substrates. The presence of these metals at the active site of the enzyme suggests a mechanistic connection between PP-1 and metal-dependent hydrolases of known structure such as 3'-5' DNA exonuclease<sup>19</sup>, alkaline and acid phosphatases<sup>20,21</sup> and certain metalloproteases, all of which use two or more metals in catalysis.

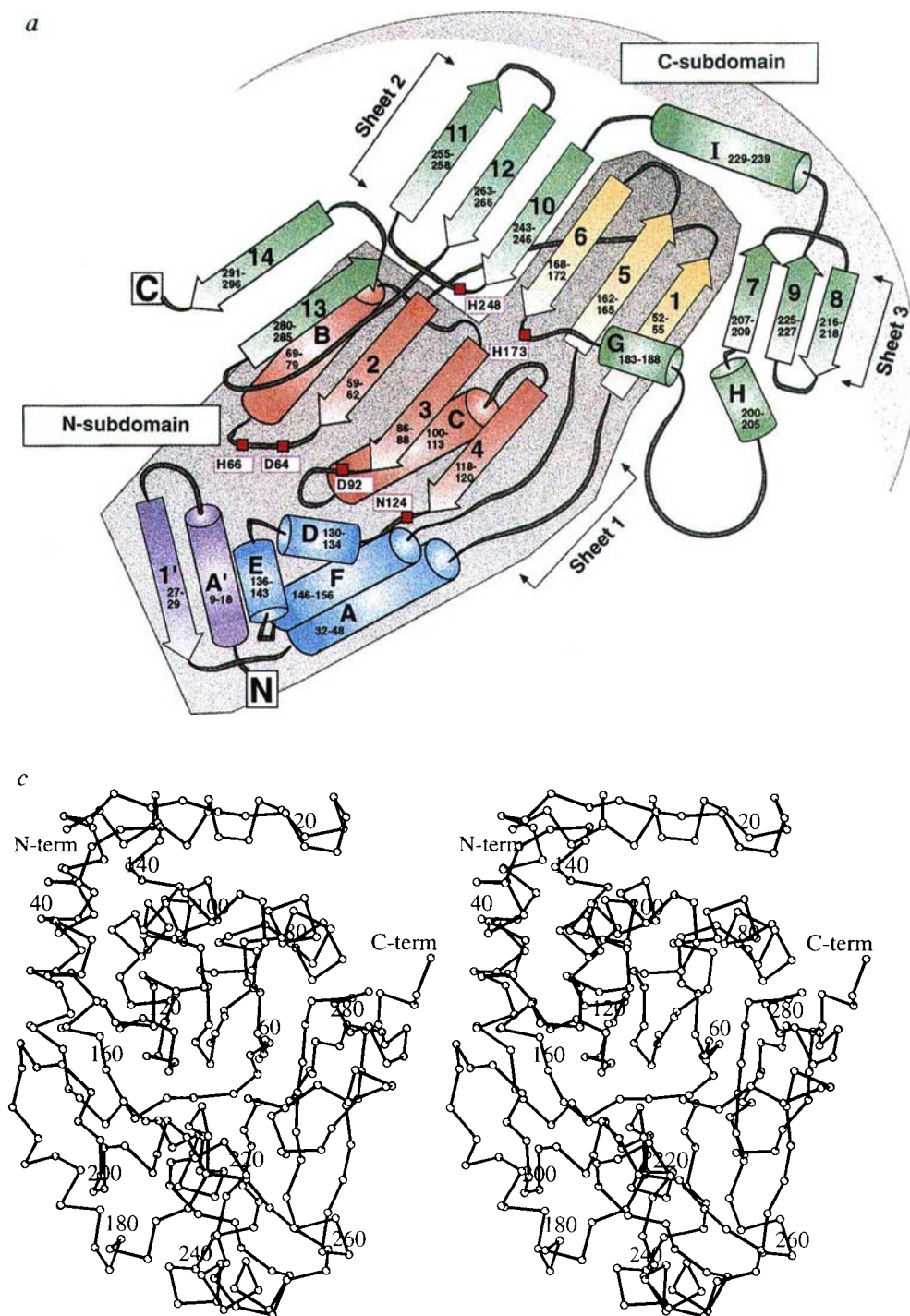
It has been uncertain whether Ser/Thr phosphatases actually require metal ions in order to function and, if so, what these metals are. The strong anomalous diffraction signal observed with Cu  $K\alpha$  X-rays (Fig. 3a) establishes the presence of two metal ions and rules out Mg, Ni, Cu or Zn in our crystals, but cannot discriminate between the presence of Mn, Fe or Co. There is evidence for Fe and Zn in calcineurin<sup>22</sup>, which has identical metal-coordinating residues. The purple acid phosphatases, which contain a similar  $\beta/\alpha$  metal-coordinating unit<sup>21</sup>, use  $\text{Fe}^{2+}/\text{Fe}^{3+}$  (mammalian) and  $\text{Fe}^{3+}/\text{Zn}^{2+}$  (plant)<sup>23</sup>. Several indirect pieces of biochemical evidence, such as the ability of Mn to restore full activity to the recombinant enzyme used in this and other studies<sup>24</sup>, as well as its presence in the crystallization buffers, suggest that Mn is the active-site ion in the crystal

sulphur atoms in the asymmetric unit. W1 and W2 (red spheres) are water molecules. *b*, Stereo view of the active site of PP-1. Metal ions 1 and 2 (purple) and waters W1, W2 and W3 (red) are drawn as spheres. Side-chain atoms of selected residues are colour-coded by atom type: nitrogen, blue; oxygen, red. Residues that are metal ligands are yellow and the geometry of their ligation to the metals is indicated by dashed white lines (site 1, square pyramidal; site 2, trigonal bipyramidal). Side chains of amino acids that are likely to be involved in catalysis are in brown. The backbone trace of the  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  scaffolding is shown in red. Note that the metal-liganding residues and catalytic residues which are contributed by the  $\beta$ - $\alpha$ - $\beta$ - $\alpha$ - $\beta$  unit make up the metallophosphoesterase motif referred to in the text. Figure was drawn using MOLSCRIPT and Raster3D. The four equatorial ligands of M1 are provided by two protein side chains (the metal-bridging oxygen atom of Asp 92 and the N $\epsilon$  atom of His 66) and two exogenous ligands (the metal-bridging W2, and a water molecule, W1). W1 donates two hydrogen bonds, one to the hydroxyl oxygen of Tyr 272 and another to an  $\alpha$ -carboxylate oxygen of the  $\gamma$ -linked D-glutamate residue of microcystin. The one axial ligand of M1 is a carboxylate oxygen of Asp 64, and thus all the protein ligands of M1 are provided by the  $\beta/\alpha$  metal-coordinating motif. M2 shares the two bridging ligands with M1, which are equatorial (W2) and axial (Asp 92) with respect to the M2 coordination. The two

other equatorial ligands of M2 are the N $\epsilon$  atom of His 173 and the carboxamide oxygen of Asn 124, a less frequently encountered metal ligand. The second axial ligand of M2 is the N $\delta$  atom of His 248. Two of the M2 ligands are from the  $\alpha/\beta$  unit (Asp 92 and Asn 124). One (His 173) is in the loop following  $\beta 6$ , which is at the carboxyl end of the N subdomain. The remaining protein ligand, His 248, is in the loop connecting strands  $\beta 10$  and  $\beta 11$ , in the C subdomain. An additional water molecule, W3, lies in the shallow active-site pocket, close to M2 but outside the coordination sphere (the W3–M2 distance is 3.4 Å). W3 makes hydrogen-bonding interactions with a number of active-site groups, including Asn 124, His 125 and W2. c, Diagram of the active site. Metal ions (M1 and M2) are coloured purple, and water molecules (W1 and W2) are green. Dashed black lines indicate the metal coordination and hydrogen-bonding interactions in the refined structure, with interaction distances (average of the two crystallographically-independent copies) also in black. Water molecule W3 is not drawn: in its place, a possible binding mode for a modelled substrate phosphate group is shown in red. The distances between the modelled substrate phosphate and active-site residues are also red. Two possible routes to a nucleophilic attack on the phosphate are indicated by red arrows. Modelling required only the removal of microcystin and W3 from the crystal structure coordinates. No changes were made to atomic positions.



FIG. 3 *a*, Schematic diagram of the secondary structure of PP-1.  $\alpha$ -Helices are shown as cylinders and  $\beta$ -strands as arrows. The lengths of the helices and strands and the loops connecting them are not to scale. The  $\beta$ - $\alpha$ - $\beta$  scaffolding that binds the two metal ions is coloured red, with the metal ligands indicated with red squares. Regions of the structure coloured violet, blue, yellow and red constitute the N-subdomain (outlined with darker grey shading) and those that make up the C-subdomain are green (outlined by the shaded arc). The separation into these two subdomains is conceptual and does not imply that they form separable structures. *b*, Schematic diagram of the three-dimensional structure of PP-1, displayed using the programs MOLSCRIPT<sup>47</sup> and Raster3D<sup>48</sup>. The secondary structural elements are coloured as in *a*. The two metal ions are shown as violet spheres. The C $\alpha$  atoms of the residues that ligate the metals are indicated by magenta circles. *c*, Stereo diagram of the C $\alpha$  atoms of PP-1 (drawn using MOLSCRIPT<sup>47</sup>). Every twentieth residue is numbered. This figure shows the molecule in a different orientation from *b*, rotated  $\sim 90^\circ$  around the viewing axis. *d*, Molecular surface of PP-1, coloured according to electrostatic potential, calculated using the program GRASP<sup>49</sup>. The electrostatic potential was calculated using a dielectric constant of 2 for the protein interior and 80 for the solvent, and assuming an ionic strength equivalent to 150 mM KCl. For the calculation, lysine and arginine residues have a net positive charge, glutamate and aspartate a negative charge and all other residues are neutral, with each of the metals assigned charges of +2. Regions of the surface that have a highly negative electrostatic potential are shown in red, with a smooth variation in colour through white (zero) to blue (positive). The microcystin molecule is drawn with nitrogen atoms in blue, oxygen atoms red and carbon atoms yellow. The locations of the two metal ions are indicated by a violet circle. The orientation of the molecule is similar to that in *b*. *e*, Potential mode of binding of the phosphothreonine and adjacent residues of DARPP-32 to PP-1. The surface of PP-1 is viewed from the same orientation as in *b* and *d*, with individual atoms shown as van der Waals spheres. Hydrogen atoms are not displayed, and the radii of carbon atoms have been increased to reflect the hydrogens. Side-chain atoms of arginine and lysine residues are shown in blue, glutamate and aspartate residues are red, and hydrophobic side chains (Ala, Val, Leu, Ile, Met, Cys, Phe, Tyr, Trp) are white; the metal ions are coloured violet and all other atoms are yellow. A possible binding mode for 12 residues of DARPP-32, including Thr 34 which is phosphorylated, is suggested by indicating the approximate locations of the side chains in the acid and hydrophobic grooves of PP-1, assuming the peptide chain of the inhibitor to be in an extended conformation. This figure is not meant to imply that a detailed docking calculation has been made, but rather to highlight the observed complementarity in the properties of this region of DARPP-32 and the surface of PP-1. **METHODS.** Refinement of the model was done with XPLOR<sup>50</sup>; non-crystallographic symmetry constraints were maintained and the resolution was extended from 2.8–2.3 Å. At 2.1 Å resolution the quality of the



model was improved by releasing the non-crystallographic symmetry constraints, as judged by the free-*R* value criterion<sup>43</sup>. The refined model contains residues 7–300 for both molecules in the asymmetric unit and a total of 277 water molecules, four Mn atoms (modelled at unit occupancy), two microcystin-LR molecules (each covalently attached at the sulphur atom of Cys 273) and two molecules of  $\beta$ -mercaptoethanol (forming mixed disulphides with Cys 291 in each PP-1 molecule). Cys 127 is oxidized. The two molecules in the asymmetric unit (A and B) are very similar, and molecule A is used for most of the analysis in this paper. The six N-terminal residues and 30 C-terminal residues in both molecules are disordered, as is the loop connecting residues 21–24 in molecule B. The average temperature (*B*) factor for all protein atoms is 20.7 Å<sup>2</sup>, with the two molecules in the asymmetric unit having average *B*-factors of 21.2 and 20.1 Å<sup>2</sup> respectively. The water molecule *B*-factors range from 7.5 to 59.1 Å<sup>2</sup>, with an average value of 27.6 Å<sup>2</sup>.





TABLE 1 Data collection and refinement statistics

| Statistics of the crystallographic analysis |                        |                                |                                         |                                                |                                      |                   |                       |
|---------------------------------------------|------------------------|--------------------------------|-----------------------------------------|------------------------------------------------|--------------------------------------|-------------------|-----------------------|
| Data set                                    | Resolution<br>(Å)      | Reflections<br>measured/unique | Completeness (%)<br>overall/outer shell | $R_{\text{merge}}$ (%)*<br>overall/outer shell | $R_{\text{iso}}$ †                   | Phasing<br>power‡ | $R_{\text{cullis}}$ § |
| MIR analysis                                |                        |                                |                                         |                                                |                                      |                   |                       |
| PHMB                                        | 20.0–2.8               | 63,483/18,077                  | 96.8/81.5                               | 11.0/28.3                                      | 0.189                                | 0.97              | 0.75                  |
| ( <i>p</i> -chloromercuribenzoate, 4 sites) |                        |                                |                                         |                                                |                                      |                   |                       |
| HgCl <sub>2</sub>                           | 15.0–3.2               | 82,174/11,626                  | 99.9/100                                | 15.8/36.5                                      | 0.403                                | 0.92              | 0.82                  |
| (5 sites)                                   |                        |                                |                                         |                                                |                                      |                   |                       |
| Overall MIR figure of merit, 0.44           |                        |                                |                                         |                                                |                                      |                   |                       |
| MAD analysis                                |                        |                                |                                         |                                                |                                      |                   |                       |
| PHMB (3 sites)                              |                        |                                |                                         |                                                |                                      |                   |                       |
| λ1 (1.018Å)                                 | 15.0–2.8               | 43,952/14,528                  | 84.6/68.8                               | 6.1/23.1                                       | 0.046                                | —                 | —                     |
| λ2 (1.009Å)                                 | 15.0–2.8               | 46,893/14,979                  | 89.1/85.5                               | 6.6/26.1                                       | 0.043                                | —                 | —                     |
| λ3 (1.005Å)                                 | 15.0–2.8               | 47,234/14,908                  | 87.1/75.0                               | 7.3/27.2                                       | —                                    | —                 | —                     |
| λ4 (0.996Å)                                 | 15.0–2.8               | 45,506/14,857                  | 87.2/80.5                               | 7.2/28.5                                       | 0.049                                | —                 | —                     |
| Overall MAD figure of merit, 0.45           |                        |                                |                                         |                                                |                                      |                   |                       |
| Native data                                 | 20.0–2.1               | 180676/38768                   | 99.5/97.2                               | 5.6/13.6                                       |                                      | —                 | —                     |
| Refinement statistics                       |                        |                                |                                         |                                                |                                      |                   |                       |
|                                             | Resolution (Å)         | Completeness (%)               | <i>R</i> -factor                        | Free <i>R</i> -factor                          | No. reflections                      |                   |                       |
| Data with <i>F</i> > 2 σ <i>F</i>           | 6–2.1                  | 96.2                           | 0.173                                   | 0.229                                          | 35697                                |                   |                       |
| All data¶                                   | 6–2.1                  | 99.6                           | 0.178                                   | —                                              | 36974                                |                   |                       |
| R.m.s. deviations                           | Bonds lengths, 0.007 Å |                                | Bond angles, 1.4°                       |                                                | <i>B</i> -values, 1.9 Å <sup>2</sup> |                   |                       |

Data collection and phase determination. All diffraction data were measured from crystals cooled at 100 K. 0.2 µl glycerol was added to the hanging drops and the crystals were equilibrated for 1 h before being mounted in nylon loops and flash-frozen in a stream of N<sub>2</sub>. Native data and derivative MIR data were collected on a Rigaku R-Axis IIC image-plate detector mounted on a rotating anode X-ray generator (MSC, Houston). All data were processed using programs DENZO and SCALEPACK (Z. Otwinowski and W. Minor, unpublished results). MAD data<sup>16</sup> were collected from a single frozen crystal at the National Synchrotron Light Source, Brookhaven National Laboratory, using HHMI beamline X4A. Reproducible derivatization was achieved by exposing the crystal to PHMB (2 mM) for 4 h and then adding a crosslinking reagent (1% (v/v) glutaraldehyde). After reaction for 1 h, the crystal was back-soaked in reservoir solution containing 10% (v/v) glycerol. For the MAD data collection the crystal was mounted with the  $c^*$  axis aligned along the rotation axis and perpendicular to the X-ray beam to allow the near simultaneous recording of Bijvoet pairs of reflections. Data collected at four wavelengths near the mercury LIII absorption edge were recorded on image plates, digitized with a Fuji scanner and processed with DENZO and SCALEPACK. The determination of phases proceeded independently for the MIR and MAD data sets; both analyses used the probabilistic approach with program MLPHARE (Z. Otwinowski, CCP4 program suite). Phase probability coefficients for MIR and MAD were then combined (overall figure of merit, 0.52) and applied to native structure factor amplitudes. The initial electron density map was improved by density modification using SQUASH<sup>44</sup> and then averaged once according to the non-crystallographic symmetry using RAVE<sup>45</sup>. The resulting map was readily interpretable and model building proceeded with the program O (ref. 46).

<sup>\*</sup>  $R_{\text{merge}} = 100 \times \sum_h \sum_i |I_{h,i} - \langle I_h \rangle| / \sum_h \sum_i I_{h,i}$

<sup>†</sup>  $R_{\text{iso}} = \sum_n |F_{\text{nat},n} - F_{\text{deriv},n}| / \sum_n F_{\text{nat},n}$ . For the MAD analysis,  $R_{\text{iso}}$  is calculated between data sets at two wavelengths. Wavelength 3 is used as the reference data set in this calculation.  $F_{\text{nat}}$ ,  $F_{\text{deriv}}$  are the native and derivative structure factor amplitudes.

<sup>‡</sup> Phasing power = r.m.s. heavy-atom structure factor/r.m.s. lack of closure.

<sup>§</sup>  $R_{\text{cullis}} = \sum_h |F_{\text{deriv}} - F_{\text{nat}}| - |F_{\text{Hcalc}}| / \sum_h |F_{\text{deriv}} - F_{\text{nat}}|$  (summed over centric reflections);  $F_{\text{Hcalc}}$  is the calculated structure factor for the heavy atoms.

<sup>||</sup> Free  $R$ -factor<sup>43</sup> was calculated with 7% of the data.

<sup>¶</sup> Intensity data (including negative observations) were converted to structure factor amplitudes with the program TRUNCATE (CCP4 suite).

ions impart a strong electrostatic effect probably underlies their major catalytic role in PP-1, as suggested for 3'-5' exonuclease<sup>19</sup>. Metal ions, by stabilizing negative charges, can make a phosphate ester more susceptible to nucleophilic attack and can also stabilize the additional charge that develops on the phosphate during the attack.

We focus next on a plausible binding mode for the phosphate and the active-site residues, rather than the substrate side chain that presents the phosphate. The two metal ions are positioned at the bottom of a shallow pocket which, based on model-building and comparison with bound microcystin, could accommodate serine, threonine, histidine and tyrosine phosphoesters. The structural basis for the preferential dephosphorylation of serine and threonine side chains requires analysis of phosphopeptide complexes of the enzyme, although we do note that the Ser/Thr phosphatases, including PP-1, can dephosphorylate phosphohistidine<sup>26</sup> and phosphotyrosine under certain conditions<sup>27</sup>.

A possible binding mode for the phosphate group of the substrate is one in which it bridges the two metals, with a phosphate oxygen liganding each metal. By displacing W3, the phosphate could provide an axial ligand to M1 and an equatorial ligand to M2. There are several potential candidates for nucleophilic attack on the phosphoryl group: the metal-bound ligands W1 and W2 (as a source of nucleophilic hydroxide ions), the imidazole ring of His 125, the carboxylate of Asp 95 or possibly the phenol ring of Tyr 272 in its ionized form. The geometry of the modelled phosphate position is consistent with an in-line attack on the phosphate by W1. The requirement for a general acid

catalyst for the leaving-group serine (or threonine) of the substrate may be met by His 125 (Fig. 2*b, c*). This histidine residue is part of a conserved active-site aspartate-histidine pair, with the formation of a hydrogen bond between a carboxyl oxygen of Asp 95 and the Nδ atom of His 125 (O–N distance of 2.8 Å; Fig. 2*b*). Mutation of the corresponding histidine to asparagine in λ phosphatase has a particularly deleterious effect on activity, decreasing  $k_{\text{cat}}$  by a factor of 10<sup>5</sup> (ref. 5). Alternatively, the reaction might proceed via a covalent phosphoryl-histidine intermediate with His 125, aligned by Asp 95, forming the attacking nucleophile. Evidence for a phosphoryl-enzyme intermediate, possibly involving histidine, has been presented for the mammalian purple acid phosphatase<sup>28</sup>, which possesses the metallophosphoesterase motif<sup>21</sup>, but so far there is little direct experimental evidence for any particular reaction mechanism for PP-1.

## Surface grooves and microcystin

The active site is situated at the bifurcation point of an extended Y-shaped groove on the surface of the molecule (Fig. 3*d*). One arm of the groove (the 'hydrophobic groove') runs between the D helix and the loop connecting αG and αH, where a patch of hydrophobic side chains, conserved in PP-2A and calcineurin, is exposed at the surface. Another groove (the 'acidic groove') is formed entirely within the C subdomain, between sheets 2 and 3, and contains a number of acidic side chains. The carboxy terminus of the domain (residue 300) is at one end of the third groove (the 'C-terminal groove'). The path of this groove, which runs from the carboxy terminus of the domain to the active site,

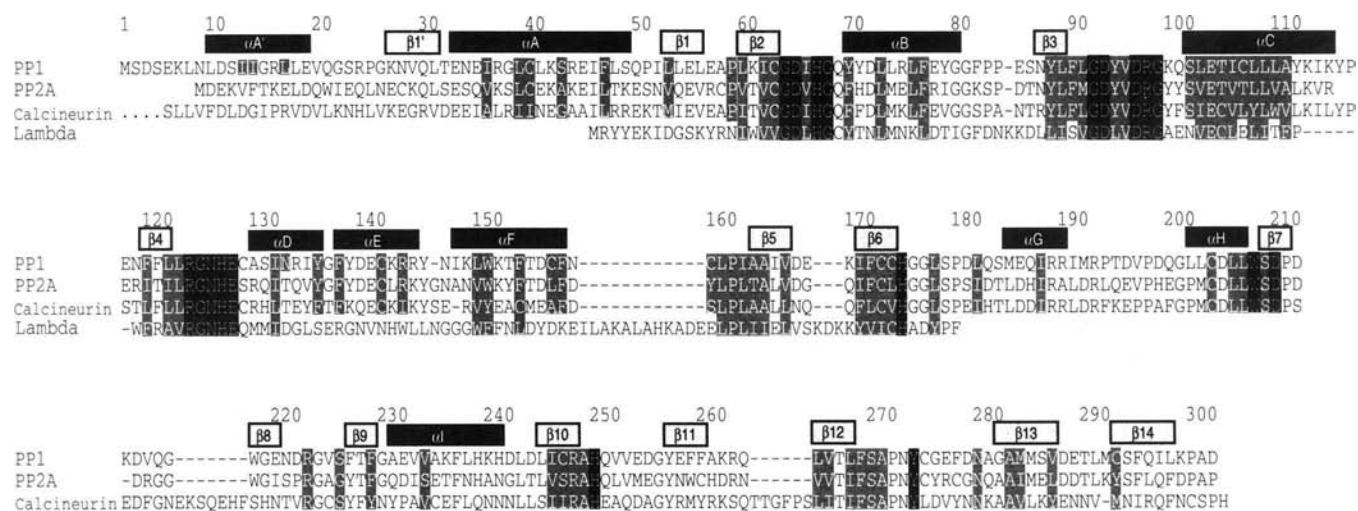


FIG. 4 Sequence alignment of Ser/Thr protein phosphatases. The amino-acid sequences of the catalytic domains of rabbit muscle PP-1<sup>36</sup>, human PP-2A<sup>37</sup>, human calcineurin<sup>38</sup>, and bacteriophage- $\lambda$  phosphatase<sup>39</sup> are shown. The sequence of the  $\lambda$  phosphatase can be aligned with that of PP-1 only in the N subdomain. The divergent C-terminal region of this enzyme is not shown. Residue numbers above the sequences correspond to PP-1. The secondary structural elements

of the PP-1 crystal structure are indicated by black boxes for  $\alpha$ -helices and grey boxes for  $\beta$ -strands. Residues that are involved in metal ligation, implicated in catalysis, or are critical to the structure of the active site in PP-1 are highlighted in black. Residues with buried side chains in the PP-1 structure are highlighted in grey. The corresponding residues in the other phosphatases are also highlighted if the hydrophobic or hydrophilic nature of the buried side chain is unchanged.

is interesting because of the inhibition of PP-1 by phosphorylation at Thr 320 (refs 8, 9). The 20-residue spacing between the site of phosphorylation and the end of the catalytic domain may be sufficient for inhibition to occur by blockage or alteration of the active site by the phosphorylated C-terminal sequence.

Microcystin binds to PP-1 in such a way as to interact with three distinct regions of the surface: the metal-binding site, the hydrophobic groove and the edge of the C-terminal groove near the active site (Fig. 5). Indirect coordination of the microcystin to the metals occurs via two of the metal-ligated water molecules, and involves a carboxylate group and a carbonyl oxygen of the toxin. The long hydrophobic Adda side chain packs into the hydrophobic groove, where it is accommodated snugly. Interactions between the microcystin and the C-terminal groove occur at the loop connecting  $\beta$ 12 to  $\beta$ 13, where the leucine side chain of microcystin packs close to the side chain of Tyr 272. We observe a covalent linkage between the toxin and a residue in this loop, involving a Michael-type addition of the  $S\gamma$  atom of Cys 273 to the terminal carbon atom of the Mdha side chain of the toxin. The covalent linkage of microcystin has been noted previously<sup>29</sup>, but is not essential for inhibition of the enzyme by the toxin, as shown by mutagenesis of the cysteine<sup>30</sup> or blockage of the reactive Mdha site in microcystin<sup>29</sup>. Another factor likely to contribute to the high affinity of microcystin for PP-1 is the close similarity of the conformation of microcystin in solution<sup>31</sup> and in complex with PP-1, which will reduce the entropy lost on binding. Further structural studies of PP-1 without bound microcystin should establish whether or not the phosphatase molecule is similarly unperturbed by inhibitor binding.

### Inhibitor-1 and DARPP-32 binding sites

The interaction of inhibitor-1 or DARPP-32 with PP-1 requires phosphorylation of a specific threonine residue in the inhibitors<sup>7</sup>, with the phosphate group of the threonine presumably binding to the active site of PP-1. The sequence surrounding this threonine in DARPP-32 (-RRRRP-pT-PAMLF-, where pT is phosphothreonine) or inhibitor-1 (-RRRRP-pT-PATLVLT-) suggests that the acidic residues around the edge of the acidic groove (Asp 210, Asp 212, Asp 220, Asp 253, Glu 252, Glu 256, Glu 275) might interact with the arginine residues preceding the phosphothreonine. The construction of a simple model for the

DARPP-32 sequence, assuming an extended chain for the peptide, indicates that the arginine side chains would be spaced appropriately to interact with negatively charged side chains of the acidic groove (Fig. 3e). Furthermore, the hydrophobic residues that follow the phosphothreonine could interact with side chains in the hydrophobic groove.

Although this analysis provides a model to explain the binding of the residues surrounding the phosphothreonine in DARPP-32 with PP-1, it does not explain why the inhibitor is not dephosphorylated by the enzyme. Crystallographic analysis of phosphopeptides bound to PP-1 should establish the extent to which DARPP-32 mimics a genuine substrate and reveal the combination of surface features responsible for interactions between enzyme and substrate.

### Discussion

Every active-site residue of PP-1 involved in metal coordination or implicated in catalysis is strictly conserved in PP-2A and calcineurin (Fig. 4). More generally, the hydrophobic or hydrophilic nature of the buried side chains in PP-1 is conserved at virtually every position in PP-2A and in calcineurin from  $\alpha$ A to  $\beta$ 14. This indicates that the tertiary structures of the enzymes will be very similar across both subdomains and, in particular, that the location of the carboxy terminus of the catalytic domain is likely to be preserved. This is of interest for calcineurin, because there is evidence that the binding of immunosuppressant/immunophilin complexes to calcineurin bridges calcineurin-B and the catalytic domain<sup>32</sup>. The binding site for calcineurin-B follows immediately after the catalytic domain of calcineurin-A, placing calcineurin-B at the end of the C-terminal groove. A mutation in yeast calcineurin-A that confers cyclosporin resistance (Thr 350 to Lys or Arg) is located within the catalytic domain, corresponding to residue 275 in the  $\beta$ 12- $\beta$ 13 loop of PP-1 (ref. 32).

The binding of the inhibitors microcystin and okadaic acid and of the protein inhibitors, inhibitor-1 and inhibitor-2, to PP-1 is mutually exclusive<sup>33</sup>. A role for the  $\beta$ 12- $\beta$ 13 loop in this inhibitory regulation is indicated by other studies of these inhibitors. Okadaic acid binds to PP-2A about 100 times more tightly than to PP-1. Replacement of residues 274-277 in the  $\beta$ 12- $\beta$ 13 loop of PP-1 (GEFD) with the corresponding residues in PP-2A (YRCCG) significantly increases the sensitivity of PP-1 to

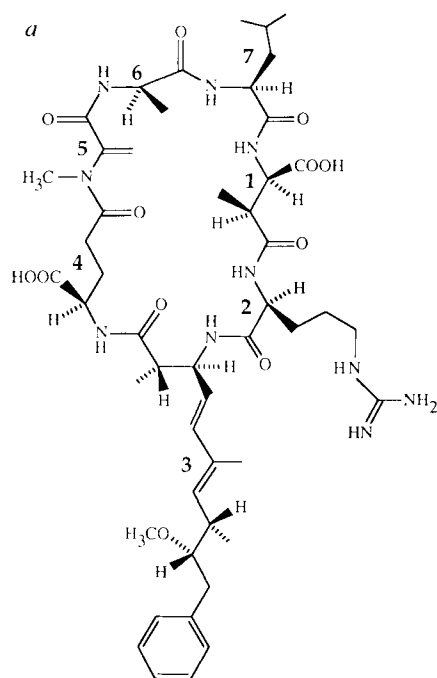
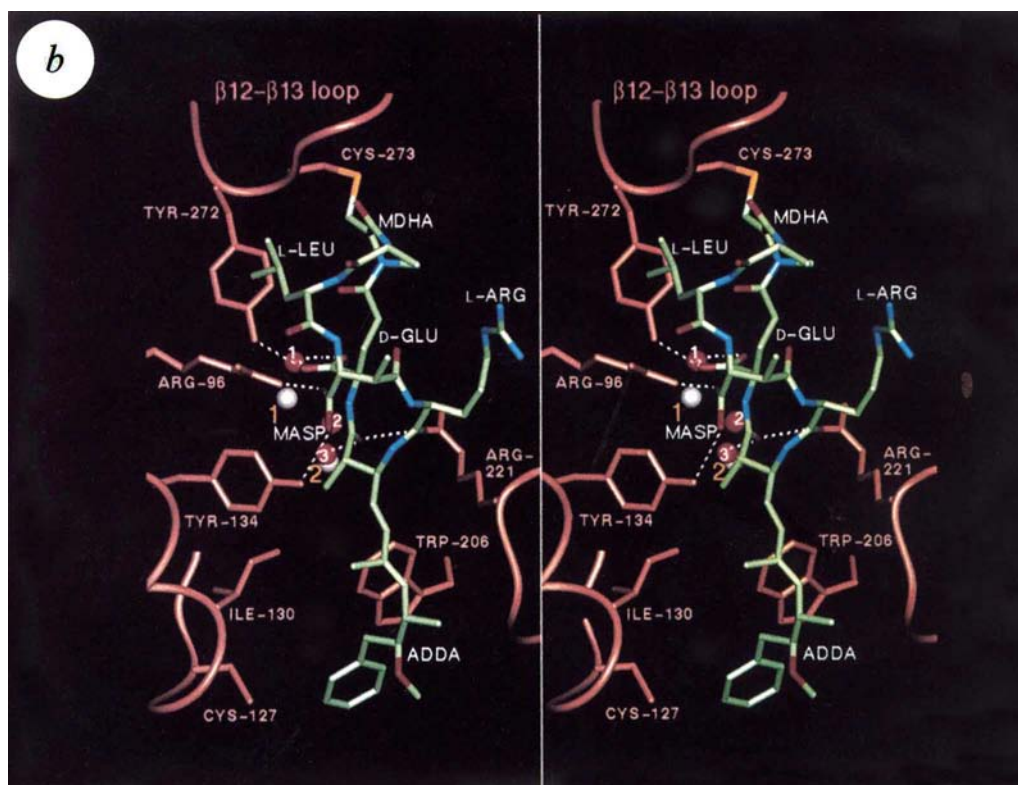


FIG. 5 *a*, Structural formula of the cyclic heptapeptide molecule, microcystin-LR<sup>31</sup>. Residues are (1)  $\beta$ -linked D-erythro- $\beta$ -methylaspartic acid (Masp); (2) L-arginine; (3)  $\beta$ -(2s, 3s, 8s, 9s)-3-amino-9-methoxy-2, 6, 8-trimethyl-10-phenyldeca-4,6-dienoic acid (Adda); (4)  $\gamma$ -linked D-glutamic acid; (5) N-methyldehydro-alanine (Mdha, the  $\beta$ -carbon of this side chain is covalently linked to Cys 273 of PP-1); (6) D-alanine; (7) L-leucine. *b*, Stereo view of microcystin-LR at the active site of PP-1. The microcystin molecule is colour-coded according to atom type: carbon, green; nitrogen, blue; oxygen, red. Main-chain and selected side-chain protein atoms are in brown; metal ions M1 and M2 (white) and waters W1, W2 and W3 (red) are drawn as spheres; the  $\gamma$ -sulphur atom of Cys 273 is yellow. The carboxylate group of the  $\gamma$ -linked D-Glu side chain and the adjacent carbonyl oxygen form hydrogen bonds with two water molecules (W1 and W3). The carboxylate group of the Masp side chain hydrogen-bonds with Arg 96 and Tyr 134. The hydrophobic Adda group packs in a groove formed by a set of hydrophobic residues. The L-Leu group lies close to the side chain of Tyr 272. The overall conformation of the microcystin is very similar to that observed in solution by NMR<sup>31</sup>. The essential chemical and conformational features of microcystin are also preserved in its active homologue nodularin<sup>31</sup>.



okadaic acid, suggesting that this region is involved in binding<sup>34</sup>. These residues are immediately adjacent to Tyr 272 and Cys 273, which interact with microcystin in PP-1. The  $\beta$ 12- $\beta$ 13 loop may also play a role in the specificity of the inhibition of PP-1 by DARPP-32. Several of the residues in PP-1 that may interact with DARPP-32 are altered in PP-2A and calcineurin. The presence of Arg in PP-2A instead of Glu 275 in PP-1 is particularly interesting as this change occurs in precisely the same region of the  $\beta$ 12- $\beta$ 13 loop that affects okadaic acid binding in PP-1 and PP-2A, and cyclosporin/cyclophilin binding in calcineurin.

In contrast to the close relationship between the Ser/Thr- and Tyr-specific protein kinases, the architecture and metal-dependent catalytic mechanism of PP-1 differ from those of the tyro-

sine phosphatases, which require no metal ions and use a cysteine nucleophile for catalysis<sup>35</sup>. The two-metal active site of PP-1 explains the function of a set of highly conserved residues and should lead to the elucidation of the reaction mechanism shared by the family. The distinctive surface features of PP-1 and an understanding of the structural basis of the inhibition of the enzyme by microcystin will provide insight into the specificity and regulation of this and other Ser/Thr phosphatases by protein inhibitors, targeting subunits and natural toxins. □

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## LETTERS TO NATURE

## Structural evidence for a two-step process in the depinning of the superconducting flux-line lattice

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A TYPE II superconductor in a magnetic field is penetrated by a hexagonal lattice of quantized flux lines. An applied current imposes a Lorentz force on these lines, but motion of the lattice will always be inhibited by pinning to material defects. Beyond a certain 'critical' current density, the lattice can break free of its pins and flow, dissipating energy and destroying superconductivity in the sample. The microscopic nature of this process is still poorly understood; in particular, little is known about the detailed structure of the flux-line lattice as it begins to depin and flow in response to the applied current. We have used small-angle neutron scattering<sup>1–3</sup> to image the structure of the flux lattice in NbSe<sub>2</sub> in the presence of a direct current, while also measuring the transport properties. Our observations of the structure of the flux lattice near the critical current verify theoretical predictions<sup>4</sup> of the existence of three regimes as a function of increasing driving force (or current): first, no motion; then disordered, plastic motion; and finally, at high velocities, a coherently moving flux crystal.

Our experiments were performed on a large (9 × 5 × 1 mm) single crystal<sup>5</sup> of 2H-NbSe<sub>2</sub>. X-ray and neutron diffraction confirmed the 2H polytype with *c*-axis mosaic <0.1° full-width at half-maximum (FWHM). Magnetization and transport studies showed a sharp (<40 mK width) superconducting transition at

7.4 K. Our experiments employed the small-angle neutron scattering (SANS) spectrometer in the cold-neutron guide hall of the DR3 reactor at Risø National Laboratory. A superconducting magnet in persistent mode produced a horizontal field parallel (to within 1°) to both the *c* axis of the crystal and the neutron beam. The samples were masked with cadmium foil to expose an area of 4 × 3 mm, covering all electrical contacts to reduce the small-angle-scattering background. In our experiment, the neutron wavelength ( $\lambda_n \approx 11.8$  Å) was fixed with bandwidth  $\Delta\lambda_n/\lambda_n \approx 18\%$ , and incident beam divergences of 0.10° and 0.27° FWHM in the horizontal and vertical directions, respectively. The diffracted neutrons were counted by an area detector located at the end of a 6-m evacuated chamber.

Three correlation lengths can, in principle, be extracted from experiments in the scattering geometry shown in Fig. 1. The two positional correlation lengths perpendicular to the flux lines  $\xi_p^{r \perp G}$  and  $\xi_p^{r \parallel G}$ , in directions, *r*, perpendicular and parallel to the reciprocal lattice vector, *G*, correspond to compressional and shear displacements of the lattice, respectively. These two lengths can be extracted from the radial and azimuthal widths of the six first-order scattering peaks which can be brought down onto the plane of the detector by appropriate rotating and tilting of the cryostat. The third length, the longitudinal correlation length  $\xi_L$ , is a measure of correlations in the lattice parallel to the direction of the flux lines. It is extracted by measuring the scattered intensity in the Bragg peaks as the sample and the magnet rotate simultaneously around an axis perpendicular to both the incoming neutron beam (*k*<sub>0</sub>) and the scattering wave-vector (*τ*<sub>10</sub>). The plot of scattered intensity versus the angle in which the flux lines are 'rocked' through the Bragg condition, forms a rocking curve. The expression  $\sigma_m^2 = \sigma_{\text{rock}}^2 - \sigma_{\text{res}}^2$  relates  $\sigma_m$ , the intrinsic FWHM rocking-curve width,  $\sigma_{\text{rock}}$ , the measured width, and  $\sigma_{\text{res}}$ , the instrumental resolution. The longitudinal correlation length  $\xi_L$  is inversely related to the rocking curve width  $\sigma_m$  by the relation  $\xi_L = 2/\tau_{10}\sigma_m = (1/\pi)((\sqrt{3}\phi_0)/(2B\sigma_m^2))^{1/2}$ , where  $\phi_0 = hc/2e$  is the superconducting flux quantum and *B* is the magnetic field. (As the rocking curves are of lorentzian shape, this definition of  $\xi_L$ , which differs by a factor of 2 from that of ref. 3, has more physical meaning.)



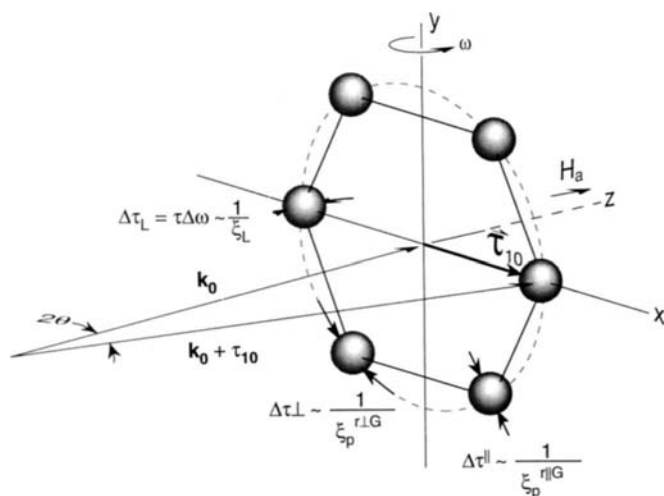


FIG. 1 The scattering geometry for our small-angle neutron scattering (SANS) experiment.

In the present study,  $\xi_p^{rLG}$  and  $\xi_p^{rG}$  are always resolution-limited with a lower limit of  $0.5 \mu\text{m}$ . For field-cooled samples, with the field between 0.2 and 3 kOe, we find that  $\xi_L$  is not resolution-limited. Previously<sup>3</sup> we were able to show, using independent measurements of the critical current density and  $\xi_L$  as a function of field, that the longitudinal correlation length varies with field in a similar fashion as the pinning length obtained from the theory of Larkin and Ovchinnikov<sup>6,7</sup>. In the present study we have much higher resolution than before, and are thus able to see the behaviour of the flux lattice near the critical current, just as it begins to unpin and flow. The improved resolution allows us to make the experimental observation that this is a two-step process.

Shown in Fig. 2 is the FWHM of the rocking curve  $\sigma_{\text{rock}}$  as a function of current, for a sample at 4.8 K which was field-cooled at 1.5 kOe and zero applied current. Figure 3 shows the differential resistivity for this sample under the same conditions. As seen on a linear scale, this differential resistivity indicates that the sample has a critical current near 1.5 A. As has been shown previously<sup>3</sup>, at the critical current the FWHM collapses to the resolution limit of our apparatus. This implies that the longitudinal correlation length  $\xi_L$  grows rapidly as the lattice depins and flows in response to the applied current as shown in Fig. 3. In the flowing regime, the lattice crystallizes with resolution-limited correlation lengths. When the lattice is repinned by removing the applied current, the flux lattice does not disorder, but retains essentially resolution-limited correlation lengths. We focus here on the behaviour of the lattice just below the critical current.

As can be seen in both Figs 2 and 3, for currents just below the critical current of 1.5 A, there is a pronounced increase of the FWHM (or a decrease of  $\xi_L$ ). Figure 3 inset shows the dependence of the longitudinal correlation length  $\xi_L$  in this region on an expanded scale. The field-cooled sample has a zero-current correlation length of  $\sim 11 \mu\text{m}$ . As the current is increased,  $\xi_L$  initially changes very little. Then, just below the critical current, the correlation length drops to  $\sim 9 \mu\text{m}$ . It finally increases to beyond the resolution limit ( $50 \mu\text{m}$ ) in the flowing state.

There is additional evidence from our SANS experiment that the plastic flow regime is separate and distinct from either the pinned-lattice or flowing-lattice states. The rocking curves in both pinned and flowing states are perfect lorentzians, as expected for exponentially decaying correlations and seen previously. But in the intermediate, plastic flow regime there is excess intensity in the tails of these curves, consistent with short-range disorder. Thus, in the structural studies, both the rocking-

curve width and the extended wings provide evidence that there are three separate regimes for the vortex lattice as a function of current.

To correlate the structural studies with vortex dissipation, we have also made sensitive transport measurements using a differential measurement scheme. This approach uses a direct current with a small ( $\sim 10 \text{ mA}$ ) superimposed alternating current. A phase-sensitive detector measures the differential resistivity (by measuring the a.c. voltage) as a function of this d.c. bias. These data at  $T=4.8 \text{ K}$  are shown in Fig. 3. The voltage (with a noise floor of  $\sim 30 \text{ pV}$ ) as a function of current can also be obtained by integrating the differential resistivity. This is shown in the log-linear plot in Fig. 2. These transport data confirm the SANS results indicating that the critical current is a two-step process. To within our voltage resolution, below  $\sim 0.8 \text{ A}$ , there is no measurable dissipation and the measured correlation length is approximately current-independent. Between 0.8 and 1.5 A, the transport is consistent with thermally activated flux creep,  $V = V_0 \exp[(J/J_c - 1)U/kT]$ , for  $T=4.8 \text{ K}$ , with a critical current  $J_c = 1.46 \pm 0.02 \text{ A}$  and  $U/kT = 7.4 \pm 0.5$ , where  $U$  is an activation barrier. In this current range, the correlation length goes through a minimum. Above 1.5 A the transport has a power-law form, as seen previously<sup>8</sup>, with  $V = V_0(J/J_c - 1)^\alpha$  where  $\alpha = 1.7 \pm 0.1$ . Here, the correlation length is resolution-limited. Data at two different temperatures (4.2 and 4.8 K) support the thermally activated form for creep, and show  $\alpha$  to be independent of temperature.  $J_c$  is, of course, temperature-dependent. It is worth pointing out that a simple d.c. transport measurement, with a voltage criterion of  $>20 \text{ nV}$  for the critical current, would suggest an abrupt critical current at 1.5 A. However, the data in Fig. 2 demonstrate that a more sensitive measurement shows voltage well below this current due to flux creep. In the limit of low temperatures, the critical current represents a sharp transition between dissipative and non-dissipative flow. But even at  $T=4.8 \text{ K}$ , there is a regime below 0.8 A ( $\sim J_c/2$ ) where both SANS and transport data show no evidence of flux motion. Although our SANS measurements of  $\xi_L$  at 1.5 kOe show that

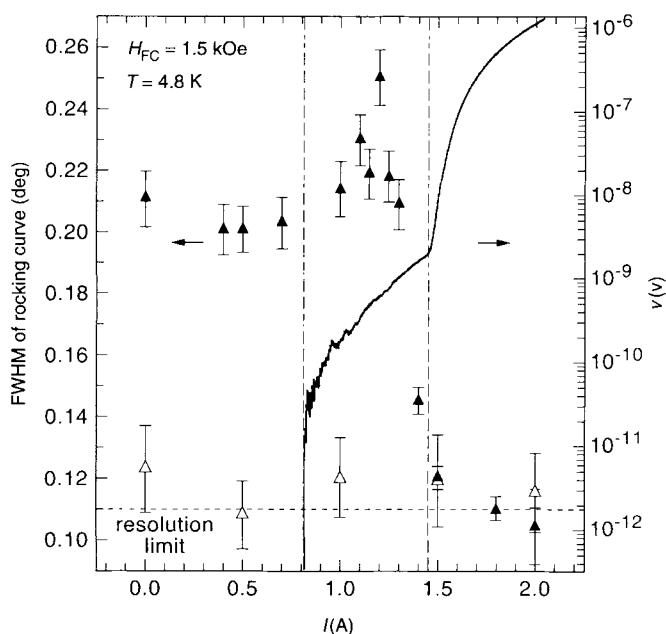


FIG. 2 The FWHM of the rocking curves for a sample cooled to 4.8 K in an applied magnetic field  $H_{FC} = 1.5 \text{ kOe}$  as a function of current  $I$  for both increasing (filled triangles) and decreasing (open triangles) currents. Also shown is the measured voltage  $V$  as a function of current. The vertical dashed lines separate the three regimes: pinned lattice, plastic motion and flowing crystal.

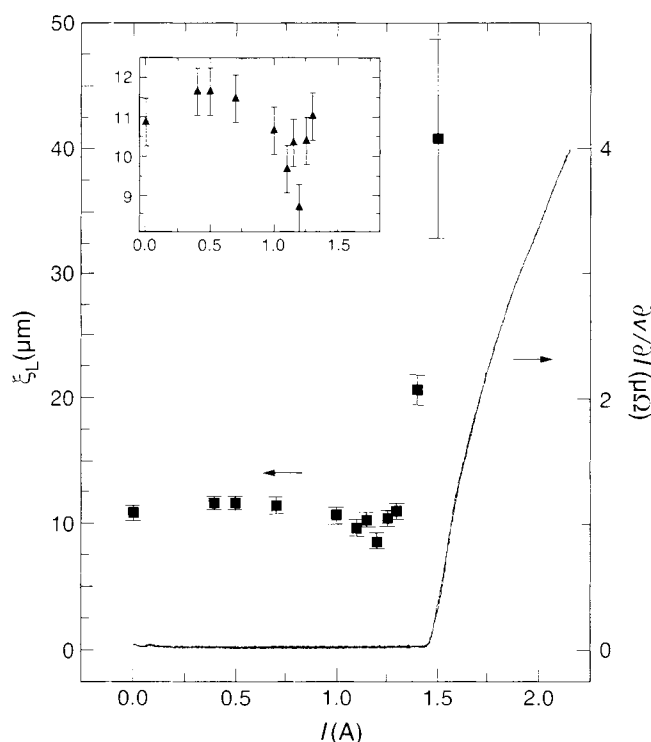


FIG. 3 The longitudinal correlation lengths  $\xi_L$  (left-hand y-axis) extracted from the rocking-curve widths and the differential resistivity (right-hand y-axis) as a function of current. Inset, the region near the critical current on an expanded scale.

correlations on length scales of 10 to 50  $\mu\text{m}$  are substantially changed by cycling the current, the transport measurements are completely reversible, with no hysteresis. This suggests that the pinning lengths that determine the dissipation, and thus the critical current, must be shorter than 10  $\mu\text{m}$  in order not to be affected by the large changes in  $\xi_L$ . At higher fields we do find hysteresis in the current-voltage curves, in agreement with previous workers<sup>9</sup>. Unfortunately, at these fields SANS does not have the resolution to measure  $\xi_L$ .

The data reported here agree with recent theoretical studies of Koshelev and Vinokur<sup>4</sup>. Using both analytical theory and numerical simulations, these workers have argued that there should be a regime of plastic flow intermediate between the pinned-lattice and flowing flux crystal. In this intermediate regime, the lattice should be more disordered than for either the moving crystal or the pinned lattice. This is in agreement with our directly measured correlation lengths. In this intermediate, plastic flow regime, we find  $I$ - $V$  curves that are well described by a flux-creep functional form. The dynamics of the superconducting flux lattice provide insight into the general class of problems involving driven elastic media in random potentials. In the flux lattice both the nature of the pinning potential and the elastic interactions of the flux lines can be manipulated. This allows one to go from the pinning-dominated regime, associated with sand piles and avalanches, to the regime of strong elasticity, where narrow-band noise is expected, as in charge density waves. The structural data presented here shed light on the specific problem of the detailed nature of such systems near the depinning threshold.  $\square$

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## Large changes in oceanic nutrient inventories from glacial to interglacial periods

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CHANGES in ocean chemistry and circulation have been invoked to explain the lower atmospheric  $\text{CO}_2$  concentrations of glacial periods observed in ice-core records<sup>1</sup>. The processes that modulate these concentrations are not well understood, but an increase in the nutrient inventory of the ocean is one mechanism that could lower atmospheric  $\text{CO}_2$  levels by enhancing oceanic biological productivity and  $\text{CO}_2$  storage<sup>1–3</sup>. The oceanic concentrations of one such nutrient, nitrate, may be regulated by changes in the rate at which it is degraded by bacteria (denitrification) in oxygen-deficient subsurface waters. Denitrification constitutes a significant global sink for oceanic nitrate<sup>4</sup>, and the eastern tropical North Pacific Ocean is particularly important in this respect as it accounts for at least a third of global oceanic fixed-nitrogen removal by water-column denitrification<sup>4,5</sup>. Here we present  $^{15}\text{N}/^{14}\text{N}$  records from marine sediment cores, which show that water-column denitrification in the eastern tropical North Pacific Ocean was greatly diminished during glacial periods. We suggest that, because nitrate limits biological productivity in much of the modern ocean, a consequent increase in the oceanic nitrate inventory during glacial periods could have contributed to the observed decrease in atmospheric  $\text{CO}_2$  concentration.

Water-column denitrification accounts for almost half (60–90  $\text{Tg N yr}^{-1}$ ) of the total oceanic loss of fixed nitrogen and occurs primarily in three areas: the eastern tropical North Pacific (ETNP), the eastern tropical South Pacific and the Arabian Sea<sup>4,6</sup>. Each of these regions is characterized by an intense oxygen-minimum zone with near-zero oxygen and high nitrate concentrations, and upwelling-induced primary production which supplies copious quantities of settling organic detritus that sustain high rates of denitrification in subsurface waters<sup>4</sup>. Denitrification rates in these zones are therefore sensitive to climate-induced changes in upwelling intensity and hydrography. In the Arabian Sea for example, Altabet *et al.*<sup>7</sup> recently concluded that denitrification was reduced substantially during glacial periods. To assess the global validity of this conclusion, we have reconstructed the history of denitrification and upwelling in the eastern tropical North Pacific for the past 140 kyr by using  $^{15}\text{N}/^{14}\text{N}$  ratios measured on bulk sediments and sedimentary proxies of biogenic fluxes from cores raised from the northwestern Mexican continental margin.

The  $^{15}\text{N}/^{14}\text{N}$  ratios in organic matter produced in the near-surface ocean are a function of the  $\delta^{15}\text{N}$  of the source  $\text{NO}_3^-$  supplied from subsurface waters and the isotopic fractionation of  $\text{NO}_3^-$  during uptake by phytoplankton<sup>8</sup> ( $\delta^{15}\text{N}$  is defined in Fig. 1 legend). In the oxygenated waters of the open ocean, the

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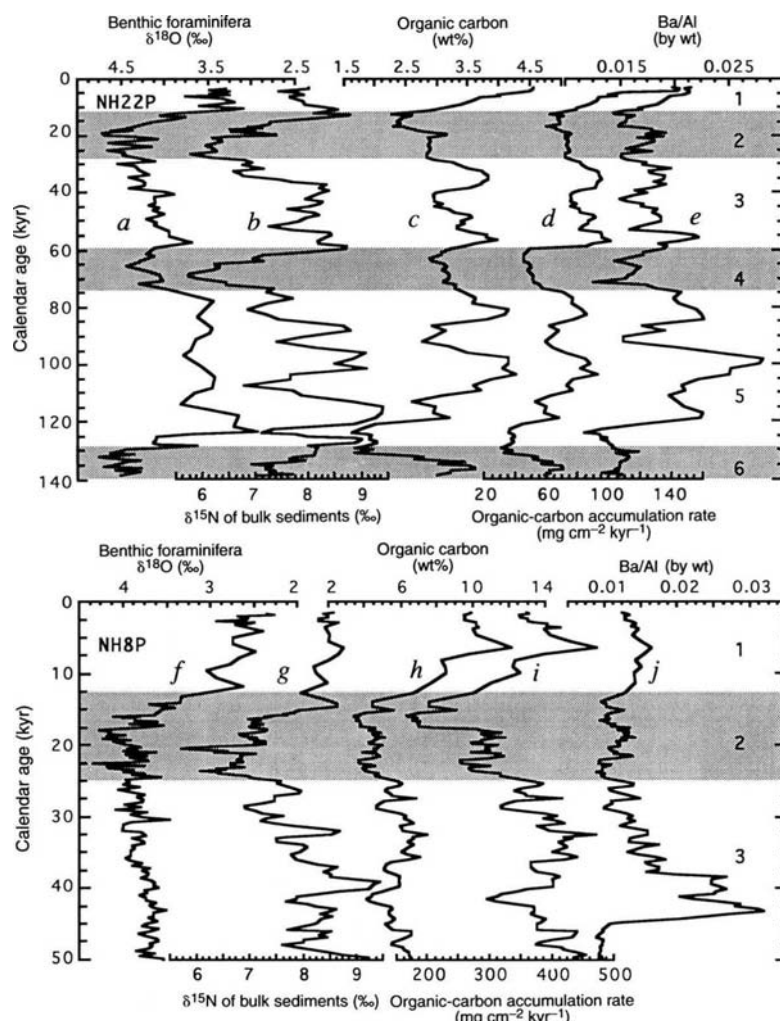
isotopic composition of the source nitrate is  $\sim 5\text{--}6\text{‰}$  (ref. 9), and therefore the  $^{15}\text{N}/^{14}\text{N}$  ratio of the organic matter produced in the euphotic zone is predominantly governed by the discrimination against  $^{15}\text{N}$  associated with phytoplanktonic assimilation of  $\text{NO}_3^-$  (ref. 8). But in oxygen-depleted waters,  $\text{NO}_3^-$  is used as an electron acceptor instead of  $\text{O}_2$  in the bacterially mediated degradation of organic matter. The gaseous products of denitrification ( $\text{N}_2\text{O}$  and  $\text{N}_2$ ) are largely lost to the atmosphere; this process constitutes a net loss of fixed nitrogen from the ocean<sup>10</sup>. As denitrification proceeds, the residual nitrate in the oxygen-deficient subsurface waters becomes progressively enriched in  $^{15}\text{N}$  because of the large fractionation factor characteristic of denitrification (1.03–1.04)<sup>11</sup>. Nitrate highly enriched in  $^{15}\text{N}$  ( $\delta^{15}\text{N}$  values in excess of +18‰) has been found in the upper water column of the ETNP<sup>11</sup>. Some of this 'heavy' nitrate is supplied to the surface waters by upwelling, which accounts for the unusual  $\delta^{15}\text{N}$  enrichments observed in suspended particulate organic material<sup>9,12</sup> and in modern bulk sediments in this area (R.S.G., unpublished data). Upwelled nutrients are completely utilized in the vicinity of the continental margin in this region<sup>13</sup>, and therefore the mean isotopic composition of both particulate organic nitrogen<sup>12</sup> and surface sediments matches that of the  $\text{NO}_3^-$  source<sup>11</sup>.

We have constructed records of variations in the intensity of denitrification in the ETNP during the Late Quaternary from measurements of bulk-sediment  $\delta^{15}\text{N}$ , organic carbon, barium and aluminium concentrations, and the  $\delta^{18}\text{O}$  of benthic foraminifera in two piston cores raised from the northwestern Mexican continental slope south of the Gulf of California (Fig. 1). The  $\delta^{15}\text{N}$  profiles (Figs 1b, g) of both cores vary in general

sympathy with the glacial-interglacial stratigraphy (Fig. 1a, f) during the past 140 kyr: heavy values (mean 8–9‰) characterize interglacials (oxygen-isotope stages 1, 3 and 5), whereas values 2–3‰ lighter occur during the glacial oxygen-isotope stages 2, 4 and 6. These glacial-interglacial variations in  $\delta^{15}\text{N}$  occur synchronously in both cores during the past 50 kyr, indicating that the signal is regional in nature. Organic-carbon/total-nitrogen ratios (by weight),  $\delta^{13}\text{C}_{\text{organic}}$  values and sedimentary lignin contents (R.S.G., unpublished data) indicate that the organic matter preserved in these sediments is of overwhelmingly marine origin, and thus variations in  $\delta^{15}\text{N}$  profiles cannot be explained by variations in the proportions of deposited terrestrial and marine organic matter. Organic-carbon concentrations and accumulation rates, as well as Ba/Al ratios (Fig. 1c–e, h–j) are all higher in the interglacial periods compared with glacial periods. High sedimentary Ba concentrations are generally accepted to be a proxy for high productivity<sup>14</sup>. The coherence between the records of organic-carbon concentration/accumulation rate and Ba/Al ratios in core NH22P imply that glacial-interglacial variations in the flux of organic carbon on the Mexican margin are a direct result of productivity variations driven by upwelling. In core NH8P, which is located in the lower portion of the oxygen-minimum, sulphate reduction in the sediments is thought to have fostered barite dissolution<sup>15</sup> and this will have diminished the glacial-interglacial Ba/Al contrast.

The heavy  $\delta^{15}\text{N}$  values (8–9‰) coincident with the high organic-carbon concentrations and Ba/Al ratios in the interglacial sediments reflect intense upwelling and the delivery to the mixed layer of  $^{15}\text{N}$ -enriched nitrate from the underlying zone of denitrification, conditions similar to those existing today in the

FIG. 1 Records of  $\delta^{18}\text{O}$  of benthic foraminifera,  $\delta^{15}\text{N}$  of bulk sediments, weight percent organic carbon, organic-carbon accumulation rate and the Ba/Al weight ratio from the northwestern Mexican continental margin. Upper panel, core NH22P (2,025 m water depth;  $22^\circ 31.1' \text{N}$ ,  $106^\circ 31.1' \text{W}$ ). Lower panel, core NH8P (1,018 m water depth;  $22^\circ 23.3' \text{N}$ ,  $107^\circ 04.5' \text{W}$ ). The timescale for ages  $< 24$  kyr is based on accelerator mass spectrometry (AMS)  $^{14}\text{C}$  age determinations corrected for reservoir age (400 yr) and calibrated to U/Th age<sup>29</sup>. Ages  $> 24$  kyr are derived from oxygen-isotope stratigraphy<sup>30</sup>. Oxygen-isotope stages are indicated near the right margin of the panels, and glacial stages are shaded grey. a, f,  $^{18}\text{O}/^{16}\text{O}$  ratios, measured on *Uvigerina* spp. in NH22P and *Bolivina* spp. in NH8P, using a VG PRISM mass spectrometer equipped with a VG Autocarb common-bath sample preparation system. The results are reported in the  $\delta$  notation,  $\delta^{18}\text{O} = [(^{18}\text{O}/^{16}\text{O})_{\text{sample}} / (^{18}\text{O}/^{16}\text{O})_{\text{standard}} - 1]$  per mil, where the standard is PDB. The analytical precision for these measurements is  $\pm 0.04\text{‰}$ . b, g, Nitrogen isotopes, measured using a Carlo-Erba CHN analyser interfaced directly to the mass spectrometer.  $\delta^{15}\text{N} = [(^{15}\text{N}/^{14}\text{N})_{\text{sample}} / (^{15}\text{N}/^{14}\text{N})_{\text{standard}} - 1]$  per mil, where the standard is atmospheric nitrogen; the precision of this measurement is better than  $\pm 0.3\text{‰}$ . c, h, Total carbon determined by Carlo-Erba CNS analyser, and carbonate carbon determined by coulometry. Organic carbon was obtained by difference with a combined precision of  $\pm 3.9\%$ . d, i, Organic-carbon accumulation rate ( $\text{mg cm}^{-2} \text{ kyr}^{-1}$ ) =  $2,400 (\text{mg cm}^{-3}) \times [1 - \text{porosity}] \times [\text{sedimentation rate} (\text{cm kyr}^{-1})] \times [\text{fraction organic carbon}]$ , where 2,400 is the assumed grain density and porosity is obtained from water-content data. e, j, Ba and Al, measured with an automated Philips PW1400 X-ray fluorescence spectrometer<sup>31</sup>. The Ba concentration is normalized to Al to minimize variability in the profile attributable to aluminosilicate-hosted barium.



ETNP. The decreases in the organic-carbon concentrations and Ba/Al ratios in the glacial-age deposits argue for a decrease in productivity and a reduced supply of organic detritus to the ocean floor, which should have resulted in a less-intense oxygen-minimum zone at these times. This process is reflected by lighter glacial  $\delta^{15}\text{N}$  values, implying that denitrification and the supply of  $^{15}\text{N}$ -enriched nitrate to the mixed layer were substantially reduced during such periods. Sedimentary  $\delta^{15}\text{N}$  values of 5–6‰ are common in areas where intermediate waters are well oxygenated and surface waters have moderate nutrient concentrations, as in much of the eastern equatorial Pacific<sup>8</sup>. In addition, sediment cores collected within the oxygen minimum on the northwestern Mexican margin exhibit laminated interglacial intervals but bioturbated glacial strata, indicating enhanced oxygenation of the water column during glacial episodes<sup>16</sup>.

Although the oxygen-minimum zone in the ETNP extends from 100 to 800 m depth near the continental margin<sup>11</sup>, intense denitrification and isotopically heavy nitrate are confined to the 100–400 m depth interval<sup>11,17</sup>. This zone coincides with the salinity maximum of the Subtropical Subsurface Water (SSW) located at around 250 m depth on the Mexican margin<sup>17,18</sup>. The source of the SSW is the Equatorial Undercurrent and these waters are well oxygenated until they reach the eastern Pacific, whereupon the oxygen content decreases rapidly as they spread north and south<sup>18</sup>. The residence time of the SSW is thought to be relatively short (~10 yr) owing to extensive upwelling in the ETNP<sup>18</sup>. Therefore the low oxygen content and consequent denitrification in these waters is attributable mainly to the high biological production (net annual primary production ~330 g C m<sup>-2</sup> yr<sup>-1</sup>; ref. 19) in surface waters off Central America. Thus, the glacial decreases in denitrification in the ETNP have probably been driven by reduced upwelling-supported biological production and fluxes of organic detritus on the continental margins, as our data indicate.

The diminished glacial-age denitrification implied by our results has profound global ramifications. Fixed nitrogen is supplied to the ocean by rivers, *in situ* nitrogen-fixation by cyanobacteria and atmospheric fallout, with the major sinks being denitrification in oxygen-deficient waters and in shelf sediments<sup>5</sup>. Previous investigations have revealed that the fixed-nitrogen budget of the ocean is unbalanced, and as a result, the ocean is losing fixed-nitrogen at a rate faster than it is being supplied<sup>6,20</sup>. The estimated supply of nitrogen to the oceans is 90 Tg N yr<sup>-1</sup> and the total loss is thought to be 175–205 Tg N yr<sup>-1</sup>, yielding a deficit of 85–115 Tg N yr<sup>-1</sup> (refs 4, 6, 21). The feedback mechanisms that could potentially modulate variations in oceanic nitrogen contents do not seem to be tightly coupled. For example, contributions by nitrogen-fixing cyanobacteria are thought to be small at present, ~20 Tg N yr<sup>-1</sup>, of which only 5–10 Tg N yr<sup>-1</sup> are fixed in open-ocean oligotrophic areas, the rest being contributed by nitrogen fixation in estuaries, marshes and coral reefs<sup>22</sup>. These observations have led to the suggestion that the oceanic nitrogen content oscillates on glacial interglacial timescales, such that the ocean gains nitrogen during times of glacial advances and loses it when glaciers retreat<sup>4,6,20</sup>.

Christensen *et al.*<sup>6</sup> have argued that the supply of nitrogen to the oceans increases during glacial advances by way of reduction in terrestrial biomass and nitrogen release from erosion of shelf sediments. The latter could contribute  $4.2 \times 10^4$  Tg N, which, if supplied uniformly over 10,000 yr, would amount to a supply-rate increase of 4.2 Tg N yr<sup>-1</sup>. The enhanced nitrogen supply resulting from a decreased terrestrial biomass is of the same order. By contrast, Christensen *et al.*<sup>6</sup> assert that the sink for nitrogen increases during interglacials owing to growth of the area of the submerged continental shelf that hosts sedimentary denitrification. Reducing the shelf area by 75%, as during the Last Glacial Maximum<sup>23</sup>, would decrease modern shelf denitrification rates from 100 (A. H. Devol, personal communication) to 25 Tg N yr<sup>-1</sup>, assuming that the decrease in denitrification is proportional to the decrease in shelf area. This 'source', when

added to the nitrogen that could be potentially derived from diminished terrestrial biomass and shelf-sediment erosion, would at best balance the glacial nitrogen budget, but the sum would be far from sufficient to cause a buildup of fixed-nitrogen in the glacial ocean. From these considerations, it is apparent that an increase in the nitrogen inventory<sup>4,6,20</sup> requires an additional decrease in the sink.

Our results from the ETNP agree remarkably well with those of Altabet *et al.* in the Arabian Sea<sup>7</sup>, suggesting that denitrification in both of these regions was reduced substantially during glacial episodes. Furthermore, studies from the eastern tropical South Pacific also show a general decrease in productivity accompanied by lighter  $\delta^{15}\text{N}$  values during the LGM<sup>24</sup>. As these three areas account for almost all of the 60–90 Tg N yr<sup>-1</sup> lost due to water-column denitrification in the present ocean, global denitrification must have been greatly diminished during glacial periods. Assuming that water-column denitrification was completely absent during the LGM and that shelf denitrification was reduced by 75%, the total annual nitrogen loss would have been reduced to ~40 Tg N yr<sup>-1</sup>, pushing the glacial oceanic-nitrogen mass balance to a substantial surplus. This scheme is consistent with previous model results<sup>25</sup> that suggest that a 75% reduction in the area of oxygen-deficient water column, is enough to increase the oceanic NO<sub>3</sub><sup>-</sup> inventory by 25%.

Primary production in the modern ocean is thought to be nitrogen-limited<sup>4,26</sup> although in some areas production may be constrained by a dearth of micronutrients such as iron<sup>27</sup>. Nitrogen has a much shorter residence time (of the order of 10<sup>3</sup>–10<sup>4</sup> yr) than phosphorus (~10<sup>5</sup> yr), and its abundance in the ocean is more likely to fluctuate on glacial-interglacial timescales<sup>4,6,20</sup>. Thus, relative to nitrogen, phosphorus does not appear to have been important as a marine productivity regulator during the Late Quaternary. Indeed the molar N:P ratios in shallow waters in large areas of the Pacific and Indian Oceans where denitrification occurs are much lower<sup>26</sup> than that predicted by the Redfield ratio (N:P = 16:1; ref. 28). Given the observed relative nitrogen-deficiency in the modern ocean, we suggest that reduced denitrification in the glacial ETNP, in concert with a similar reduction in the northern Arabian Sea and eastern tropical South Pacific, may have played an important role in lowering glacial atmospheric CO<sub>2</sub> levels by enhancing oceanic productivity in areas that are today considered to be oligotrophic. By this mechanism, a significant increase in the glacial NO<sub>3</sub><sup>-</sup> budget could in theory account for much of the observed *p*CO<sub>2</sub> decline seen in the ice-core records<sup>2</sup>. □

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## Laboratory investigation of the interaction of off-axis mantle plumes and spreading centres

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MANTLE plumes and mid-ocean ridge spreading centres are the dominant phenomena through which mass and heat are transported from the mantle to the Earth's surface. It now seems that the dispersion of near-ridge plumes beneath the lithosphere is modulated strongly by mid-ocean ridges; in particular, geochemical and geophysical observations have suggested that rising plumes are diverted towards and feed nearby ridges<sup>1–7</sup>. Here we confirm the feasibility of this model with laboratory experiments that incorpo-

rate the essential physical and fluid dynamic aspects of a plume-ridge upper mantle system. Our results indicate that an off-axis plume may communicate thermally and chemically with a spreading ridge through a narrow, sub-horizontal conduit instead of a broader, radially spreading plume head. A necessary condition for this communication is the presence of a lithospheric or rheological boundary layer that thickens away from the ridge axis owing to conductive cooling. Interestingly, we find that for high plume temperatures, increasing the plume thermal buoyancy may inhibit rather than enhance plume-ridge interaction, as a result of increased erosion of the overlying lithosphere.

Recent laboratory<sup>8</sup> and numerical experiments<sup>9</sup> have considered the dynamics of plume-ridge interaction for the ridge-centred case; however, the difficult question remains as to how a plume and a ridge interact thermally, chemically and dynamically when the plume is located off axis. A model of sub-horizontal pipe-like flow from the off-axis plumes to a ridge axis along the base of the rigid lithosphere has been suggested<sup>2</sup> (Fig. 1a). Geochemical studies<sup>10–16</sup> and two-dimensional numerical experiments<sup>17–19</sup> support this channel-flow model and suggest that geochemical communication may persist over long periods of time and plume-ridge separation distances as high as 1,200 km (ref. 16). This laboratory experimental project is the first fully three-

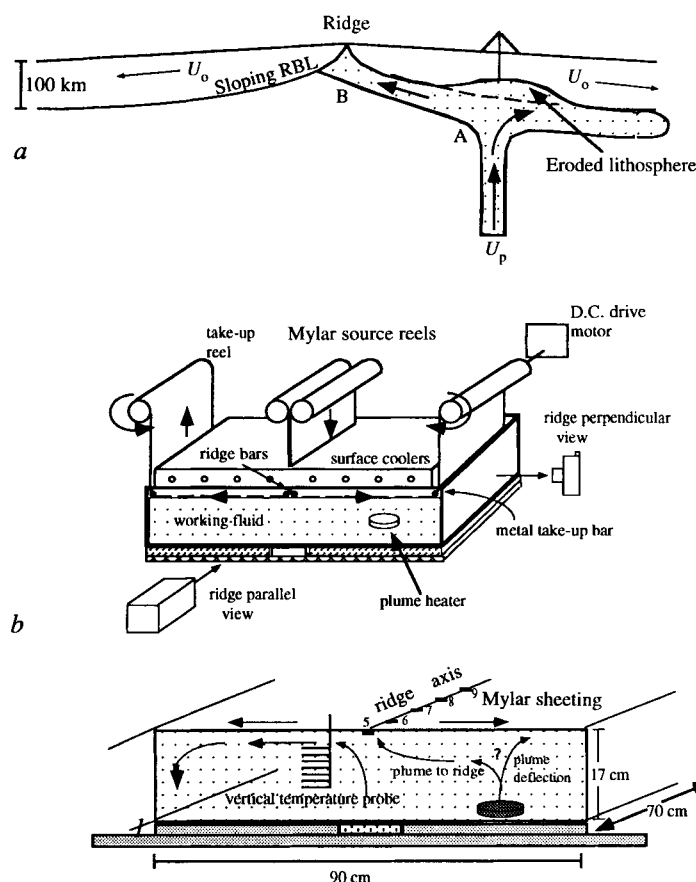
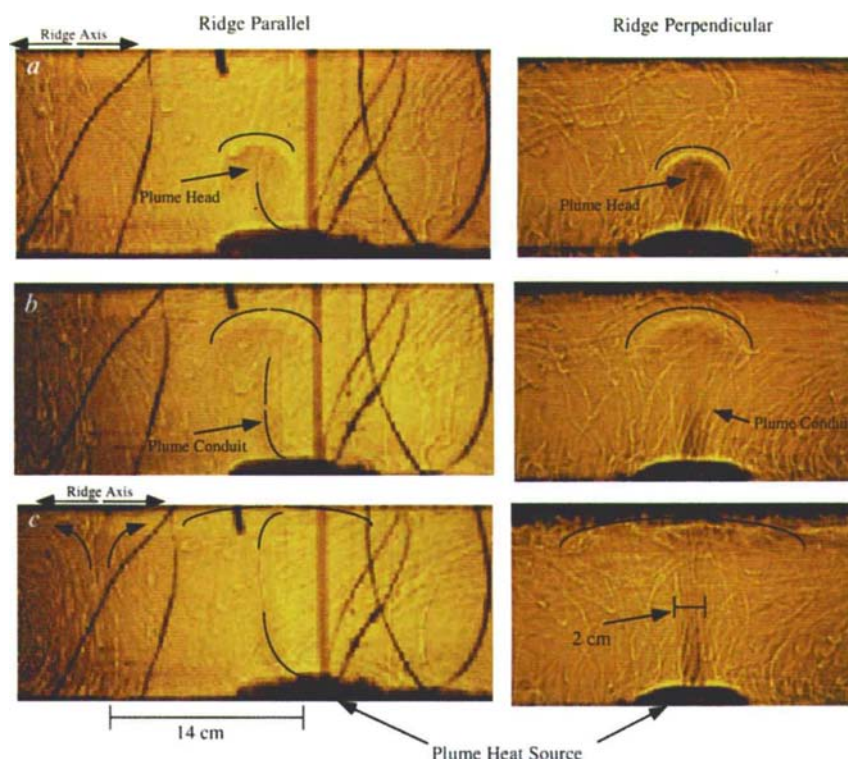


FIG. 1 a, This diagram illustrates the conceptual model that near-ridge plumes rise and then flow toward ridges along the base of the RBL from A to B (ref. 16).  $U_o$  is vertical velocity within the plume conduit. b, Diagram of experimental apparatus. Mylar sheeting is pulled along the fluid surface to simulate plate spreading. From source reels, the Mylar is threaded through two bars at the spreading axis and at the tank edges (take-up bars) to ensure contact between sheeting and working fluid. Mylar is then pulled around take-up bars by a synchronous high-torque d.c. motor. The fluid surface is cooled by circulating fluid from a refrigerated cold bath through a series of (70 cm × 10 cm × 2 cm) metal jackets suspended 2 mm above the fluid. Tank sidewalls and fluid surface are insulated. Plume flow is monitored with shadowgraph and time-lapse laser photographs from ridge-perpendicular and ridge-parallel viewpoints. Working fluid viscosity follows an Arrhenius-type law of the form  $\mu = \exp [(1,888/(T + 93.3)) - 11.48]$ , where  $T$  is temperature in °C and  $\mu$  is viscosity (in Pa s). c, This close-up slice through the tank centre illustrates the configuration of the ridge axis and plume source. Arrows illustrate hypothetical fluid flow. Nine RTDs are positioned at 5-cm intervals along the ridge axis to monitor axial temperature variability (note that ridge RTD 5 lies on an orthogonal line from the plume source). Before running experiments, we allow the fluid to equilibrate for several days at room temperature, ~20 °C. We then establish large-scale plate-driven flow by running the Mylar drives for 60 min before heating the plume source. In experiments 2 and 4, surface coolers are maintained at 10 °C for 60 min to produce an RBL before activating the Mylar; in experiment 3, coolers are maintained at 0 °C for 120 min before activating the Mylar. Experiments run for 150–280 min, depending on whether or not there is surface cooling.

FIG. 2 Shadowgraph photographs at three instants during experiment 1. Variations in fluid-temperature gradients focus the illuminating light to yield a bright halo at the top of the plume head (beneath black curves). a, 31 min after turning on the disc heater, the plume has separated from the source at the base of the tank, generating a single plume characterized by a broad leading head (~6 cm across) and a narrow trailing conduit (~1.8 cm wide). b, At 41 min, the plume begins impinging upon the fluid surface. The maximum ridgeward deflection due to the plate-driven return flow is ~2.2 cm, or roughly a conduit width. The ascent rate of the plume head is ~0.8 cm min<sup>-1</sup>, or close to twice the plate speed. Fluid velocity in the conduit exceeds 3 cm min<sup>-1</sup> as measured by tracking neutrally buoyant Delrin beads (not shown) released periodically at the plume source. c, At 72 min, the plume head has stalled and flattened; head and conduit are being sheared away from the ridge near the fluid surface.



dimensional variable-viscosity study of this problem, and it exposes the mechanisms by which an off-axis plume overcomes the lithospheric drag that draws material away from a ridge, to successfully feed the nearby spreading centre.

To test the conceptual model of plume-ridge channelling (Fig. 1a), we simulate a plume-ridge, upper mantle system with a tank of a concentrated sucrose solution which, like the Earth's mantle, is strongly temperature dependent (Fig. 1b). Plate-driven mantle flow is simulated by dragging two Mylar sheets in opposite directions on the surface of the fluid at a constant rate of 0.35 cm min<sup>-1</sup> ( $U_o$ , half-spreading rate). Buoyantly driven flow is produced through a supply of thermal energy from a disc-shaped resistance heater (that is, the plume source) positioned at the base of the tank. The two parameters we vary are the

surface temperature, thus the thickness/age of the upper rheological boundary layer (RBL), and the plume source temperature, controlling the strength of the rising plume. Fluid temperature is continuously monitored at the disc heater, at the surface of the Mylar, along a vertical profile within the fluid, and along the ridge axis with resistance temperature detectors (RTDs) (Fig. 1c).

Plume buoyancy is caused by density reduction of the plume fluid due to thermal expansion according to

$$\Delta\rho = (\rho_o - \rho_p) = \alpha\rho_o(T_p - T_o) \quad (1)$$

where  $\alpha$  is the expansion coefficient ( $4.6 \times 10^{-4}$ ), and  $\rho_o$ ,  $T_o$  and  $\rho_p$ ,  $T_p$  are reference and plume densities and temperatures,

TABLE 1 Plume-ridge experiments

| (a) Parameters                                                    |                                                   |                 |                                               |                                                 |                                              |                              |                                           |
|-------------------------------------------------------------------|---------------------------------------------------|-----------------|-----------------------------------------------|-------------------------------------------------|----------------------------------------------|------------------------------|-------------------------------------------|
| Exp. no.                                                          | Surface temp. contrast from ambient of 20 °C (°C) | RBL slope (deg) | Plume source temp. contrast from ambient (°C) | Plume density contrast, $\Delta\rho/\rho_o$ (%) | Mean plume conduit viscosity, $\mu_p$ (Pa s) | Plume buoyancy number, $B_n$ | Plume buoy. flux to ridge*, $B_r/B_s$ (%) |
| 1                                                                 | 0                                                 | <b>0</b>        | 40                                            | 1.8                                             | 4.9                                          | 52                           | 0                                         |
| 2                                                                 | -6                                                | <b>1</b>        | <b>40</b>                                     | 1.8                                             | 4.9                                          | 52                           | 3                                         |
| 3                                                                 | -10                                               | <b>3</b>        | 40                                            | 1.8                                             | 4.9                                          | 52                           | 10                                        |
| 4                                                                 | -6                                                | 1               | <b>48</b>                                     | 2.2                                             | 2.5                                          | 60                           | 0                                         |
| (b) Comparison of laboratory and expected mantle parameter ranges |                                                   |                 |                                               |                                                 |                                              |                              |                                           |
|                                                                   | $\mu_l/\mu_o$                                     | $\mu_o/\mu_p$   | $\Delta\rho/\rho_o$ (%)                       | $B_n$                                           | $Pe_p$                                       | $Pe_p/Pe$                    |                                           |
| Laboratory                                                        | 10                                                | 50-100          | 1.8-2.2                                       | 50-60                                           | 1,750-2,500                                  | 10                           |                                           |
| Mantle                                                            | >10 <sup>4</sup>                                  | ~100            | ~0.5-1.5                                      | 5-60                                            | 10 <sup>3</sup> -10 <sup>4</sup>             | 1-100                        |                                           |

\* Calculated as a ratio of plume buoyancy flux at the ridge,  $B_r = \rho_o \alpha U_o D_{rbl} \int (T(x) - T_o) dx$ , where the  $x$  axis is along the ridge,  $D_{rbl}$  is mean RBL thickness and  $T(x)$  is along-axis temperature, and plume source buoyancy flux<sup>22</sup>,  $B_s = \rho_o \alpha \Delta T U_p \pi (d/2)^2$ , where  $U_p$  is bead velocity within the plume conduit and  $d$  is conduit diameter (1.5-1.8 cm).  $B_s$  ranges between 0.003-0.004 g s<sup>-1</sup>. Reference density,  $\rho_o$ , at ambient temperature (20 °C) is 1.4 g cm<sup>-3</sup>. Important parameters for comparing results on  $B_r/B_s$  are highlighted in bold. Comparisons are also made using plume Peclet number,  $Pe_p = U_p D/\kappa$ , and the ratio  $Pe_p/Pe$ . Mantle  $Pe_p$  is calculated from Whitehead and Luther's<sup>22</sup> equation for  $U_p$ .  $B_n$ ,  $Pe_p$  and  $Pe_p/Pe$ , which best represent the vigour of plume convection relative to plate-driven flow, and  $\mu_o/\mu_p$  scale well with expected mantle values.  $\mu_l/\mu_o$  and  $\Delta\rho/\rho_o$  reflect differences in laboratory and mantle material properties.

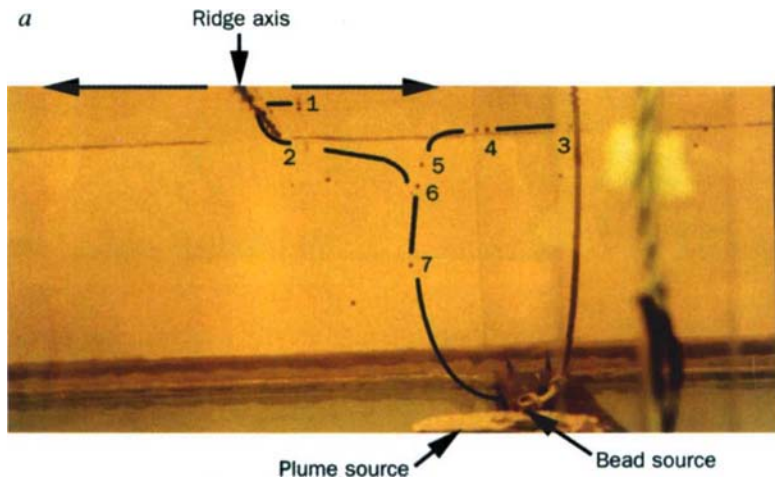
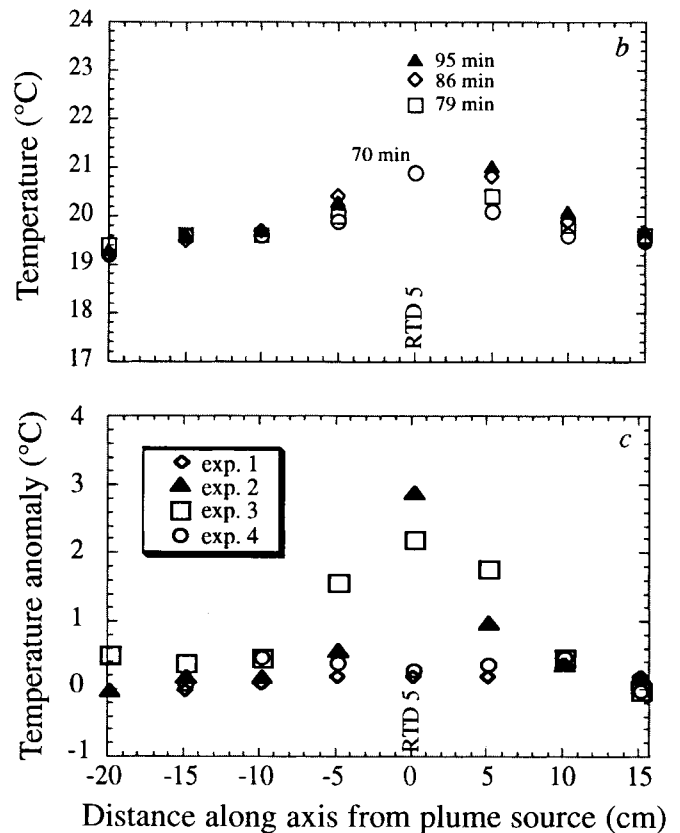


FIG. 3 *a*, Photograph of the tank fluid during experiment 2 showing locations of 7 Delrin beads (trajectories sketched in black). Bead no. 1 reached the high-viscosity upper boundary layer then travelled along a sub-horizontal path to the ridge axis similar to the depiction in Fig. 1*a*. This photograph was taken 65 min after plume initiation, 50 min after the plume–RBL impact, and 15 min after bead no. 1 hit the ridge. The bead is now frozen into the migrating plate. The photograph is taken from a mid-depth fluid level, and the dark sloping line is the ridge axis (line of RTDs) as viewed from below, through the fluid. The bead below and to the left of no. 7 was introduced while setting the bead source and is not part of the experiment. *b*, Fluid temperatures measured by eight RTDs along the ridge axis at different times during experiment 2, showing evolution of a narrow axial anomaly. Temperatures increase with time primarily at RTD 5, peaking at  $\sim 3^\circ\text{C}$  above ambient temperature. Temperatures are still increasing at 95 min, or 45 min beyond bead no. 1's arrival. *c*, Fluid temperatures along the ridge axis at the conclusions of the four experiments. Without the RBL (experiment 1) or if the plume is too hot (experiment 4), the plume fails to reach the ridge and enhance ridge temperatures. Note the broader axial anomaly for the case with a larger ( $3^\circ$ ) RBL slope (experiment 3).



respectively. We characterize the strength of the plume by the dimensionless buoyancy number<sup>8</sup>,  $B_n$ , defined as

$$B_n = \left( \frac{\Delta \rho g Q}{\mu_o U_o^2} \right) \quad (2)$$

where  $g$  is the acceleration due to gravity,  $\mu_o$  is reference dynamic viscosity and  $Q$  is the volumetric plume flux<sup>20</sup>, which is a constant  $0.14 \text{ cm}^3 \text{ s}^{-1}$  between experiments. Characterizing plumes in this manner enables us to compare plumes quantitatively between experiments (see Table 1) and provides a measure of how well our laboratory plumes represent Earth examples. Our laboratory  $B_n$  are near the upper limit of the expected range for the Earth of 7–59 (ref. 8).

Length and time scales in the laboratory models are related to the mantle through the Peclet number,

$$Pe = U_o D / \kappa \quad (3)$$

Thermal diffusivity,  $\kappa$ , of the laboratory fluid is  $0.001 \text{ cm}^2 \text{ s}^{-1}$  and the corresponding mantle value is  $0.01 \text{ cm}^2 \text{ s}^{-1}$ . We define the length scale,  $D$ , as the thickness of the laboratory fluid (17 cm) corresponding to 600 km of the upper mantle. Thus, our  $0.35 \text{ cm min}^{-1}$  Mylar speed yields  $Pe = 100$  and scales to a slow mantle full-spreading rate of  $\sim 1 \text{ cm yr}^{-1}$ . Likewise, our laboratory plume–ridge separation distance of 14 cm scales to a mantle distance of  $\sim 500 \text{ km}$ .

The four experiments we present here are selected from a total of nine to highlight the relative roles of surface cooling (compare experiment 1 with 2, and 2 with 3) and plume source temperature

(compare experiments 2 and 4) on plume–ridge communication (see Table 1). In experiment 1 (Fig. 2), the fluid surface is not cooled, but rather maintained at room temperature; therefore no sloping RBL is present. As the rising plume reaches the surface, the plume head flattens and widens (10–12 cm wide), but we see that without a sloping RBL the plume is strongly deflected away from the spreading axis such that no plume material reaches the ridge (Fig. 2c).

Experiment 2 is performed with the same plume source temperature but with surface cooling, which, combined with plate spreading, produces a characteristic upper thermal/rheological boundary layer that slopes towards the spreading axis. If we arbitrarily define the RBL as the isosurface along which viscosity is twice that of the ambient fluid ( $175 \text{ Pa s}^{-1}$ ) then the RBL thickness above the plume source is 0.25 cm (that is,  $350 \text{ Pa s}^{-1}$  at  $15.5^\circ\text{C}$ ) after the plate-driven flow has been established. The initial RBL slope in this experiment is then  $1^\circ$ , assuming zero RBL thickness at the ridge axis 14 cm away.

The role of the RBL on plume–ridge interaction is apparent in the paths of neutrally buoyant tracer beads (Fig. 3a). Most beads are deflected by the plate flow, but two (1 and 2 in Fig. 3a) migrate toward the ridge, indicating successful plume–ridge communication. The bead source is positioned on the side of the plume heater, away from the ridge axis. Because of this, and the fact that only a percentage of the plume is channelled to the ridge, a large number of beads (3–7) track the fraction of plume being deflected from the ridge. Long-term sampling of plume material by the ridge is more clearly recorded by the axial temperatures, which increase steadily with time as hotter plume material reaches the ridge (Fig. 3b). This plot shows axial temperatures still increasing at 95 min, which is 45 min (or 55 Myr) beyond the arrival of bead 1 at the ridge. Spatially, the temperature anomaly is centred on RTD 5 with an axial width of roughly 10 cm (350 km). This narrow, confined axial anomaly indicates that rather than spreading radially along the RBL, the off-axis plume is channelled rideward through a narrow conduit as predicted from constructional volcanism<sup>2</sup> and geochemical studies<sup>11</sup>. The scaled anomaly width of roughly 350 km is the approximate width of geochemical anomalies along the Mid-Atlantic Ridge (MAR)<sup>11</sup> associated with the Ascension and Tristan hotspots, both of which are  $\sim 400$  km from the MAR, similar to scaled laboratory plume–ridge offsets of 500 km.

Also consistent with the behaviour of the Tristan system is the substantial cooling of the laboratory plume as it migrates from the source to the ridge. The laboratory plume source temperature is  $35\text{--}40^\circ\text{C}$  higher than the ambient temperature. The equivalent mantle plume temperature excess is  $\sim 500^\circ\text{C}$  using a mantle  $\alpha$  value of  $3 \times 10^{-5}$ , but at the ridge the temperature anomaly is only  $\sim 3^\circ\text{C}$ , or a mantle equivalent of  $45^\circ\text{C}$ , indicating that substantial conductive cooling of plume material occurs between the source and ridge axis. Most cooling probably occurs along the sub-horizontal plume conduit (path A–B in Fig. 1a) where bead velocities drop to  $0.5\text{--}1 \text{ cm min}^{-1}$  and where the plume is in direct contact with the cold upper boundary layer.

Analogously, the Tristan plume is expected to have cooled by as much as  $200^\circ\text{C}$  along its migration path to the MAR<sup>7</sup>.

In experiment 3, the surface is cooled even further to yield an initial RBL slope of  $\sim 3^\circ$  between the plume and ridge. Here the resulting axial temperature anomaly is broader ( $\sim 20$  cm or 700 km) than with the  $1^\circ$  slope (Fig. 3c). Assuming that the axial anomaly reflects directly the plume flux to the ridge, we estimate that the rideward plume buoyancy flux (see Table 1) is  $\sim 10\%$  of the entire source flux, or  $\sim 3$  times that estimated for experiment 2, where RBL slope was a third as large. These results suggest that the proportion of a plume that feeds a nearby ridge depends directly on the amplitude of the RBL slope, which is consistent with results from two-dimensional numerical experiments<sup>18</sup>. Because the lithosphere probably slopes by  $3\text{--}10^\circ$ , even larger proportions of plumes may feed ridges in the mantle.

Finally, experiment 4 is identical to experiment 2, except with a 20% higher plume–ambient-temperature contrast. Although intuitively it might seem that increasing plume buoyancy should increase plume flow along the sloping RBL to the ridge, the absence of any plume signal at the ridge for this stronger plume case (Fig. 3c) indicates that this relationship does not necessarily hold. Instead, the hotter plume erodes a pocket in the RBL (Fig. 1a) which traps the plume head in the translating viscous plate, a result consistent with two-dimensional numerical experiments (C.K., J.-G. Schilling and C.G., manuscript submitted). The trailing conduit is tilted away from the ridge, thereby prohibiting further rideward flow. Such behaviour may, in part, explain why only the weakest plumes discussed by Sleep<sup>21</sup> have recognizable signatures at nearby ridges<sup>16</sup>. The most robust present-day plume is Hawaii, but it is located  $>5,000$  km from the nearest ridge. The prediction from these experimental results is that lithospheric erosion may have prohibited communication of the Hawaiian plume with the East Pacific Rise throughout its existence, even during 50–70 Myr ago when it was closer to the ridge than it is today.

Our fully three-dimensional variable-viscosity laboratory experiments indicate that the preservation of an upper rheological boundary that thins toward the ridge axis is the primary requirement for communication between an off-axis mantle plume and a nearby spreading centre. The greater the RBL slope, the more effectively it diverts the buoyant low-viscosity plume material to the ridge. But extremely hot plumes may erode the RBL, thus precluding the plume from feeding the ridge. Our laboratory results show that the plume signal at the ridge is narrow, indicating that communication could be through a confined conduit such as has been proposed as a result of previous observations<sup>2,11</sup>. Continuing laboratory experiments are investigating the effects of larger RBL slopes and wider ranges in plume buoyancies and plate velocities on plume–ridge dynamics. It is important to note that in cases where ridges have migrated away from ridge-centred plumes, the dynamics of interaction may be affected by the track of thin lithosphere (e.g. thermal groove<sup>16</sup>) that is left behind. To address this issue, the laboratory apparatus is also being employed in a study of stationary plumes interacting with migrating ridges and plumes beneath ridge-transform offsets. □

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# Genetic mapping of floral traits associated with reproductive isolation in monkeyflowers (*Mimulus*)

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SPECIATION is the process whereby populations acquire sufficient genetic differences to become reproductively isolated<sup>1</sup>. Since Darwin it has been recognized that the tempo and mode of speciation are greatly influenced by the number and magnitude of genetic changes required for reproductive isolation<sup>2-6</sup>, but detailed genetic studies have been limited to a few taxa such as *Drosophila*<sup>7</sup>. Genome mapping techniques now widely adopted in plant<sup>8,9</sup> and animal<sup>10,11</sup> breeding make it possible to investigate the genetic basis of reproductive isolating mechanisms in natural populations. Here we use this approach to map eight floral traits in two sympatric monkeyflower species that are reproductively isolated owing to pollinator preference by bumblebees or hummingbirds. For each trait we found at least one quantitative trait locus accounting for more than 25% of the phenotypic variance. This suggests that genes of large effect can contribute to speciation.

*Mimulus lewisii* (Fig. 1a) is bumblebee-pollinated and has pale pink flowers with contrasting yellow nectar guides, a wide corolla opening with forward-thrust petals to provide a landing platform, a small volume of high concentration nectar, and inserted anthers and stigma. *M. cardinalis* (Fig. 1c) has typical hummingbird-pollinated flowers, with red petals lacking nectar guides, a narrow tubular corolla, a large volume of nectar, and anthers and stigma exerted to make contact with the hummingbird's forehead. *M. cardinalis* is likely to be derived from a bumblebee-pollinated ancestor<sup>12,13</sup> such as *M. lewisii*. Despite the striking

difference in morphology between the two species, they are completely interfertile when artificially mated<sup>14</sup>. The F<sub>1</sub> hybrid (Fig. 1b) is vigorous and fertile; when self-pollinated it gives rise to F<sub>2</sub> segregants of astonishing diversity in floral colour and form (Fig. 1d-i). Hybrid plants have never been reported in nature in areas of sympatry, despite intensive efforts to find them<sup>14</sup>. As both species flower abundantly and continuously for several months in the late spring and summer when hummingbirds and bumblebees are active, it is clear that differences in floral morphology are sufficient to ensure reproductive isolation. Quantitative trait loci (QTLs) governing floral morphology are thus candidate 'speciation genes'<sup>6</sup>.

In conjunction with their work on adaptive genetic variation in natural plant populations, Hiesey *et al.* concluded that most floral characters in the *Mimulus* F<sub>2</sub> are controlled by a 'multiple series of genes rather than by a single gene'<sup>14</sup>. Their genetic inferences were limited, however, by the lack of mendelian markers and linkage maps. We have extended their results by objectively quantifying such variables as flower colour, adding new traits such as nectar volume, and using molecular markers and genome maps to locate QTLs affecting floral traits.

Linkage maps of *M. lewisii* and *M. cardinalis* were constructed (Fig. 2a) and used to search for QTLs controlling three classes of floral traits in the F<sub>2</sub>: pollinator attraction, reward and efficiency. Traits likely to be involved in pollinator attraction include flower colour, shape and size<sup>15,16</sup>. Pollinator reward was investigated by measuring nectar volume and concentration. Efficiency traits, that is, characters influencing pollen removal and deposition, were stamen and pistil length.

The major floral pigments in the two species are the pink and magenta anthocyanins and the yellow carotenoids<sup>12</sup>. *M. lewisii* petal lobes are pale pink because of a low anthocyanin concentration, and the carotenoids are confined to a pair of yellow nectar guides that extend down the corolla tube (Figs 1a and 3a, b). This pattern of colours is visible to bees and is typical of bee-pollinated flowers<sup>17</sup>. The red colour of *M. cardinalis* flowers results from high concentrations of anthocyanins and carotenoids jointly distributed throughout the petals (Figs 1c and 3a, b). Red flowers are attractive to hummingbirds but outside the colour vision spectrum of bumblebees<sup>17,18</sup>. A QTL on linkage

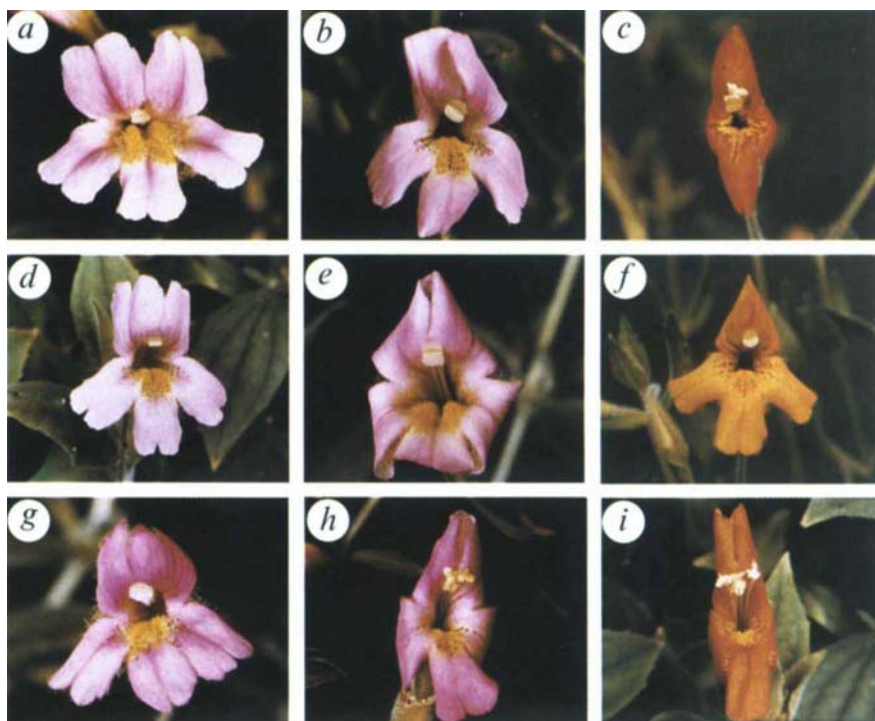


FIG. 1 *Mimulus lewisii* (a) was crossed with *M. cardinalis* (c) to produce the F<sub>1</sub> (b), and a single F<sub>1</sub> plant was self-pollinated to yield the F<sub>2</sub> (d-i). *M. lewisii* and *M. cardinalis* seeds (collected in Yosemite National Park, California), were the generous gift of Bob Vickery (University of Utah). The *M. lewisii* seed came from Raisin Lake (seedlot 13357, elevation 2,123 m) and the *M. cardinalis* from Wawona (seedlot 13363, elevation 1,300 m).

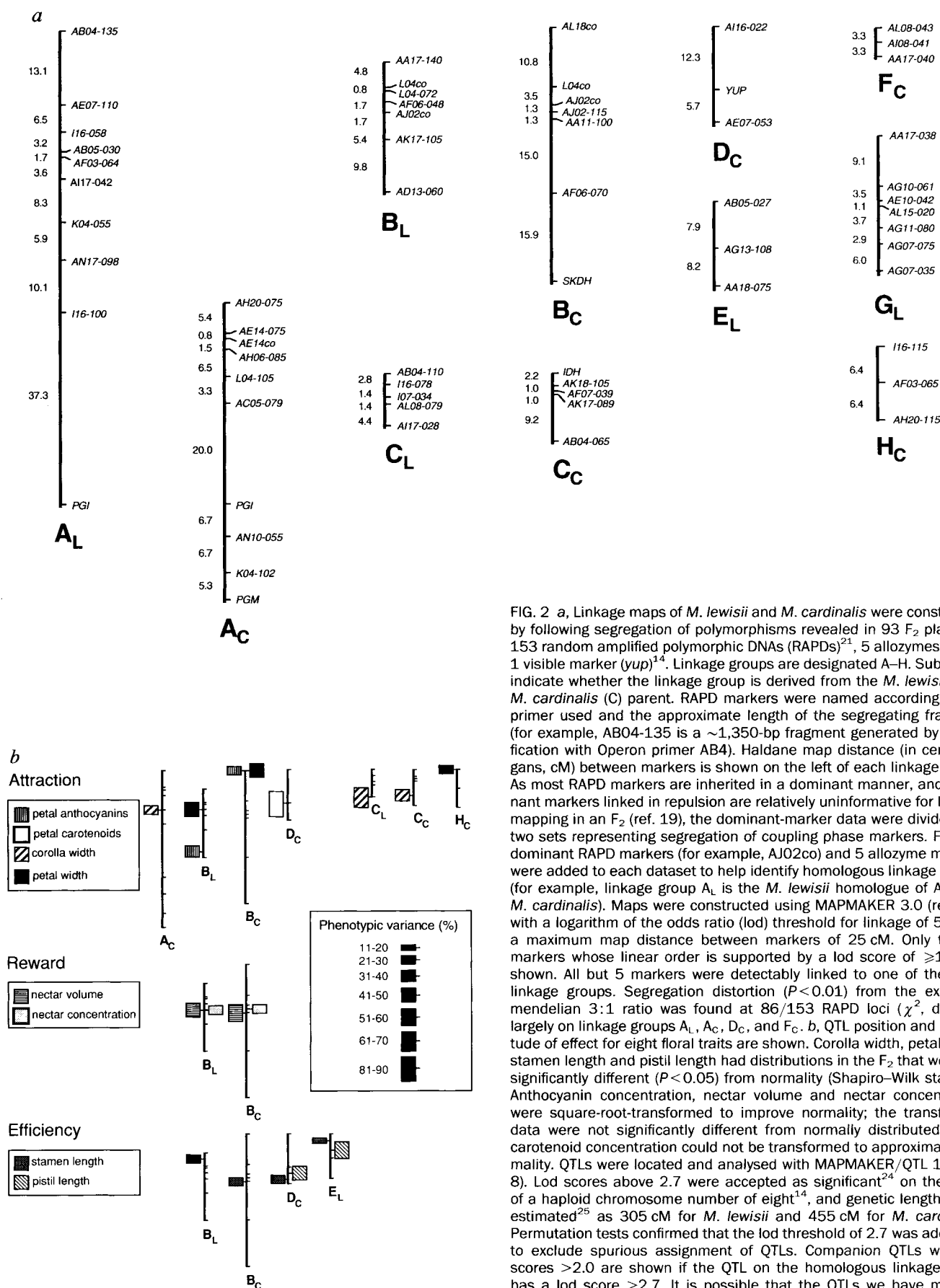


FIG. 2 **a**, Linkage maps of *M. lewisii* and *M. cardinalis* were constructed by following segregation of polymorphisms revealed in 93  $F_2$  plants by 153 random amplified polymorphic DNAs (RAPDs)<sup>21</sup>, 5 allozymes<sup>22</sup>, and 1 visible marker (*yup*)<sup>14</sup>. Linkage groups are designated A–H. Subscripts indicate whether the linkage group is derived from the *M. lewisii* (L) or *M. cardinalis* (C) parent. RAPD markers were named according to the primer used and the approximate length of the segregating fragment (for example, AB04-135 is a ~1,350-bp fragment generated by amplification with Operon primer AB4). Haldane map distance (in centimorgans, cM) between markers is shown on the left of each linkage group. As most RAPD markers are inherited in a dominant manner, and dominant markers linked in repulsion are relatively uninformative for linkage mapping in an  $F_2$  (ref. 19), the dominant-marker data were divided into two sets representing segregation of coupling phase markers. Five co-dominant RAPD markers (for example, AJ02co) and 5 allozyme markers were added to each dataset to help identify homologous linkage groups (for example, linkage group  $A_L$  is the *M. lewisii* homologue of  $A_C$  from *M. cardinalis*). Maps were constructed using MAPMAKER 3.0 (ref. 23), with a logarithm of the odds ratio (lod) threshold for linkage of 5.0 and a maximum map distance between markers of 25 cM. Only the 59 markers whose linear order is supported by a lod score of  $\geq 1.3$  are shown. All but 5 markers were detectably linked to one of the eight linkage groups. Segregation distortion ( $P < 0.01$ ) from the expected mendelian 3:1 ratio was found at 86/153 RAPD loci ( $\chi^2$ , d.f. = 1), largely on linkage groups  $A_L$ ,  $A_C$ ,  $D_C$ , and  $F_C$ . **b**, QTL position and magnitude of effect for eight floral traits are shown. Corolla width, petal width, stamen length and pistil length had distributions in the  $F_2$  that were not significantly different ( $P < 0.05$ ) from normality (Shapiro–Wilk statistic). Anthocyanin concentration, nectar volume and nectar concentration were square-root-transformed to improve normality; the transformed data were not significantly different from normally distributed. Petal carotenoid concentration could not be transformed to approximate normality. QTLs were located and analysed with MAPMAKER/QT1.1 (ref. 8). Lod scores above 2.7 were accepted as significant<sup>24</sup> on the basis of a haploid chromosome number of eight<sup>14</sup>, and genetic lengths were estimated<sup>25</sup> as 305 cM for *M. lewisii* and 455 cM for *M. cardinalis*. Permutation tests confirmed that the lod threshold of 2.7 was adequate to exclude spurious assignment of QTLs. Companion QTLs with lod scores  $> 2.0$  are shown if the QTL on the homologous linkage group has a lod score  $> 2.7$ . It is possible that the QTLs we have mapped are composed of two or more linked genes. Fine-structure mapping of individual QTLs in advanced generations of hybrid progeny will be required to resolve this issue<sup>26</sup>.

TABLE 1 Location, magnitude of effect, and mode of action for floral QTLs

| Class      | Trait                                                  | Linkage group  | Lod   | Phen% | Phen mean LL/CC | Dominance*  |
|------------|--------------------------------------------------------|----------------|-------|-------|-----------------|-------------|
| Attraction | Petal anthocyanin (A <sub>510</sub> )                  | B <sub>L</sub> | 3.32  | 33.5  | 0.63/0.79       | L†          |
|            | Petal anthocyanin (A <sub>510</sub> )                  | B <sub>C</sub> | 2.69  | 21.5  | 0.63/0.74       | add†        |
|            | Petal carotenoids (A <sub>450</sub> ) ('yellow upper') | D <sub>C</sub> | 9.59‡ | 88.3  | 0.04/0.88       | <b>L</b>    |
|            | Corolla width (mm)                                     | A <sub>C</sub> | 3.50  | 25.7  | 29.3/20.9       | add         |
|            | Corolla width (mm)                                     | C <sub>L</sub> | 4.51  | 68.7  | 33.2/18.1       | add         |
|            | Corolla width (mm)                                     | C <sub>C</sub> | 3.12  | 33.0  | 30.4/20.8       | add         |
|            | Petal width (mm)                                       | B <sub>L</sub> | 8.14  | 42.4  | 10.0/12.5       | <b>C</b>    |
|            | Petal width (mm)                                       | B <sub>C</sub> | 7.78  | 41.2  | 10.1/13.1       | <b>C</b>    |
|            | Petal width (mm)                                       | H <sub>C</sub> | 4.27  | 25.2  | 11.5/13.3       | <b>L</b>    |
| Reward     | Nectar volume (µl)                                     | B <sub>L</sub> | 10.03 | 48.9  | 6.9/32.6        | <b>add†</b> |
|            | Nectar volume (µl)                                     | B <sub>C</sub> | 10.17 | 53.1  | 6.6/32.6        | <b>add†</b> |
|            | Nectar concentration (Brix%)                           | B <sub>L</sub> | 2.83  | 23.9  | 31.9/18.7       | <b>add†</b> |
|            | Nectar concentration (Brix%)                           | B <sub>C</sub> | 3.06  | 28.5  | 32.8/18.6       | <b>add†</b> |
| Efficiency | Stamen length (mm)                                     | B <sub>L</sub> | 4.52  | 27.7  | 29.4/34.7       | <b>C</b>    |
|            | Stamen length (mm)                                     | B <sub>C</sub> | 4.46  | 27.5  | 29.9/35.4       | add         |
|            | Stamen length (mm)                                     | D <sub>C</sub> | 3.41  | 21.3  | 32.1/37.0       | <b>L</b>    |
|            | Stamen length (mm)                                     | E <sub>L</sub> | 2.92  | 18.7  | 30.3/34.1       | <b>C</b>    |
|            | Pistil length (mm)                                     | D <sub>C</sub> | 5.10  | 43.9  | 32.5/40.2       | add         |
|            | Pistil length (mm)                                     | E <sub>L</sub> | 6.93  | 51.9  | 31.8/39.2       | add         |

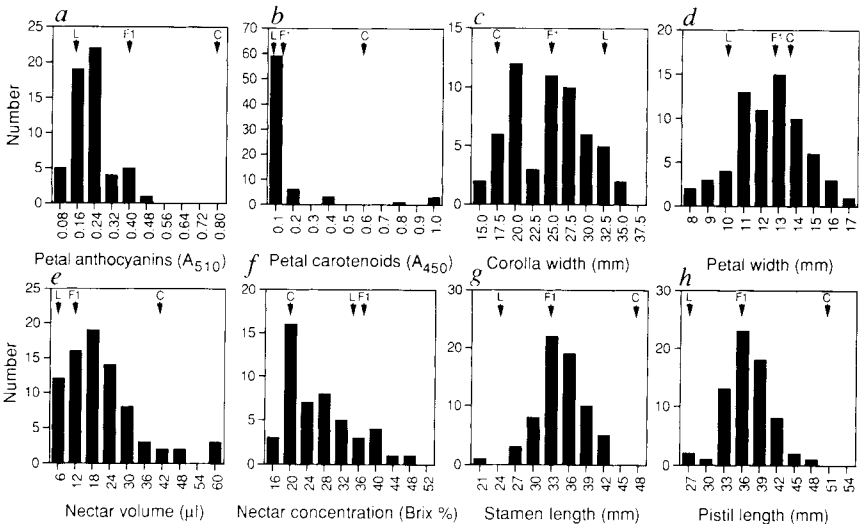
Traits were measured as described for Fig. 3 and QTLs were mapped as described in Fig. 2b legend. 'Linkage group' refers to the map in Fig. 2a; 'Lod' is the maximum lod score for each QTL; 'phen%' is the proportion of phenotypic variance explained by the QTL; and 'phen mean' is the phenotypic mean of F<sub>2</sub> plants homozygous for the *M. lewisii* (LL) or *M. cardinalis* (CC) allele at each QTL. 'Dominance' indicates whether the *M. lewisii* (L) or *M. cardinalis* (C) allele is dominant, or if the mode of QTL action is additive (add). Concordance between estimates of per cent phenotypic variance explained the magnitude of difference between homozygous classes, and mode of QTL action on homologous linkage groups was very good, in spite of the fact that dominant genetic markers provide most of the framework of the maps. The only large discrepancy is for corolla width on linkage group F, which may be explained by the relative lack of informativeness of dominant markers<sup>19</sup> or by violations of the assumption that genetic variance not due to the QTL on F is a random normal variable<sup>20</sup>.

\* Mode of QTL action is shown in bold if it has a lod support of ≥1 above competing models of dominance or additivity.  
† Mode of QTL action calculation is based on square-root transformation of the phenotypic measurements.  
‡ 'Yellow upper' was scored as a qualitative trait, and this lod score is that between *yup* and the nearest linked RAPD marker.

group B explains 21.5–33.5% of the phenotypic variance in extractable anthocyanin (Fig. 2b), with the *M. cardinalis* allele having the expected effect of increasing the anthocyanin concentration (Table 1). The presence of carotenoids in the petal lobes is governed by a single locus<sup>14</sup>, *yup* (for yellow upper; Fig. 2a, b), with the *M. cardinalis* allele recessive (Table 1). The existence of *yup* explains the highly skewed distribution of petal carotenoid concentration in the F<sub>2</sub> (Fig. 3b). Two independent QTLs affect corolla width (Fig. 2b and Table 1), a measure of the petal reflexing which converts the wide *M. lewisii* flower to a narrow tubular hummingbird flower (Fig. 3c). Petal width was included as an indicator of flower size

(Fig. 3d); two major QTLs were identified (Fig. 2b and Table 1). *M. cardinalis* produces 80-fold more nectar than *M. lewisii* (40 µl as compared with 0.5 µl per flower; Fig. 3e), and half the phenotypic variance in nectar volume in the F<sub>2</sub> is controlled by a single QTL on linkage group B (Fig. 2b, and Table 1). The *M. cardinalis* allele increases nectar volume, as expected of a hummingbird-pollinated plant, but simultaneously decreases nectar concentration (Figs 2b and 3f; Table 1). *M. lewisii* and *M. cardinalis* are highly diverged for stamen and pistil length (Fig. 3g, h). Genetic analysis of stamen and pistil length shows that these traits are partly independent, with a major QTL for stamen length on linkage group B that is not shared with

FIG. 3 a–h, Distributions of trait values for eight floral characters in the F<sub>2</sub> are shown, along with the mean values for *M. lewisii* (L), *M. cardinalis* (C), and the F<sub>1</sub>. Petal anthocyanin concentration was estimated by punching 6-mm disks from the lateral petals of two flowers per plant, extracting the anthocyanins with 0.5 ml methanol/0.1% HCl, and determining the absorbance at 510 nm. Petal carotenoid concentration was estimated similarly, using methylene chloride for extraction and measuring absorbance at 450 nm. The remaining traits were measured on two flowers per plant. Corolla width was measured with the flowers in their natural position across the widest lateral dimension. Petal width was measured on a lateral petal in its natural position. Nectar volume was measured with a graduated pipette tip. Nectar concentration (per cent soluble solids; Brix%) was determined with a hand-held refractometer. Stamen and pistil length were measured with digital calipers from the base of the calyx to the centre of the anther on the longest stamen or to the cleft in the stigma, respectively.



pistil length (Fig. 2b and Table 1). Other QTLs for the two traits are shared on linkage groups D<sub>C</sub> and E<sub>L</sub> (Fig. 2b). In every case, the *M. cardinalis* allele increases the length of the stamen or pistil (Table 1).

Our mapping experiments show that for each of eight floral traits likely to play a role in reproductive isolation there is at least one major QTL accounting for more than 25% of the phenotypic variance (Fig. 2b and Table 1). This finding suggests that the evolution of reproductive isolation may involve genes of large effect and therefore that speciation may occur rapidly.

The floral syndrome associated with hummingbird pollination is found in 18 families and 39 genera in the flora of western North America, and in many cases has evolved from bee-pollinated ancestors<sup>13</sup>. One plausible scenario for the initial steps in the evolution of hummingbird pollination in *Mimulus* would include a

sequence of three major mutations affecting pollinator attraction, reward and efficiency. A mutation at the *yup* locus causes carotenoid pigment deposition throughout the flower, reducing attractiveness to bumblebees by eliminating contrast between the petals and nectar guides. A second mutation at the major 'reward' QTL leads to greatly increased nectar volume and visitation by hummingbirds. A third mutation at the major QTL for pistil length improves the efficiency of pollen deposition by hummingbirds. This hypothesis for the evolution of hummingbird pollination is testable in part by introgressing the *M. cardinalis* allele at each major QTL into a *M. lewisii* genetic background (singly and in combination), followed by assessment of pollinator visitation and its fitness consequences in nature. □

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## A reaction–diffusion wave on the skin of the marine angelfish *Pomacanthus*

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IN 1952, Turing proposed a hypothetical molecular mechanism, called the reaction–diffusion system<sup>1</sup>, which can develop periodic patterns from an initially homogeneous state. Many theoretical models based on reaction–diffusion have been proposed to account for patterning phenomena in morphogenesis<sup>2–4</sup>, but, as yet, there is no conclusive experimental evidence for the existence of such a system in the field of biology<sup>5–8</sup>. The marine angelfish, *Pomacanthus*, has stripe patterns which are not fixed in their skin. Unlike mammal skin patterns, which simply enlarge proportionally during their body growth, the stripes of *Pomacanthus* maintain the spaces between the lines by the continuous rearrangement of the patterns. Although the pattern alteration varies depending on the conformation of the stripes, a simulation program based on a Turing system can correctly predict future patterns. The striking similarity between the actual and simulated pattern rearrangement strongly suggests that a reaction–diffusion wave is a viable mechanism for the stripe pattern of *Pomacanthus*.

When juveniles of *Pomacanthus semicirculatus* are smaller than 2 cm long, they have only three dorsoventral stripes (Fig. 1a). As they grow, the intervals of the stripes get wider proportionally until the body length reaches 4 cm. At that stage, new

stripes emerge between the original stripes (Fig. 1b). As a result, all the spaces between the stripes revert to that of the 2-cm juvenile. New lines are thin at first, but gradually get broader. When the body length reaches 8–9 cm, an identical process is repeated (Fig. 1c).

The reaction–diffusion system used here consists of two hypothetical molecules (activator and inhibitor) which control the synthesis rate of each other. Fig. 1d shows a computer simulation of a reaction–diffusion wave on a growing array of cells. At time 0, the field width is adjusted to be twice the intrinsic wavelength, calculated from the equations used in this simulation. One of the five cells is forced to duplicate periodically. As the field enlarges, all waves widen evenly. When the field length reaches about twice the original length, new peaks appear in the middle of the original peaks, as observed in *P. semicirculatus*, and the wavelength reverts to that of the original.

The juvenile of *P. imperator* has concentric stripes, which increase in number in a manner similar to that of *P. semicirculatus*. But when the *P. imperator* becomes an adult, the stripes become parallel to the anteroposterior axis by a process of continuous cutting and joining of the lines (data not shown). As they grow, the number of lines increases proportionally to body size, and the spaces between the lines are kept at an even width. The stripe pattern of *P. imperator* usually contains several branching points (Fig. 2a). During growth, the branching points move horizontally like a zip, resulting in addition of new lines. Figure 2b–d shows a branching point moving in the anterior direction until it fuses with the border of the stripe region. In Fig. 2h–l, two branching points meet and disappear leaving a new line. This type of rearrangement also happens in the simulation of the reaction–diffusion system, by setting a homologous conformation as a starting pattern (Fig. 2e–g, m–q). In Fig. 2e, the field height is adjusted to be six times the intrinsic wavelength. The waves in the right half are slightly extended, which



causes loss of stability in this region. The rightward movement of the branch restores the stability of the righthand region. It is notable that not only the final conformation, but also each intermediate stage (Fig. 2*n-p*), look quite similar to the actual pattern change that occurs in the fish (Fig. 2*i-k*).

Branching points located on more dorsal or ventral regions behave differently. As shown in Fig. 3*a-c*, they move vertically by switching at a joint. This phenomenon can be simulated by the program used in Figs 1 and 2 only by setting a different starting pattern (Fig. 3*d-f*). In the simulation, a local region that contains a branching point is less stable than a region without a branch point, and joint switching tends to occur. The direction of joint switching is determined by the conformation of neighbouring lines. In our simulation, the line under the branching point is straighter than the line above. Because the curving line is less stable than the straight line, the joint switches in the upper direction. If both upper and lower lines are symmetrical to the branched line, horizontal movement of the branch point occurs (Fig. 2). In the case of actual young fish, the lines in the middle region are usually straight, but in the dorsal and ventral regions the lines are curved. Branching points always move farther away from the middle region which consists of straight lines.

The times required for these pattern changes also suggest a mechanistic homology between actual fish and the simulations. In the simulation of joint switching, one change of joint can take place very quickly (in less than 1,000 iterations of calculation), because the change in pattern is quite local. For the horizontal movement of the branching point (from Fig. 2*e* to *g*), more than 50,000 iterations are required because it is necessary for the

upper lines and lower lines to 'slide' in order to evolve to a new pattern of stripes that are evenly spaced. In the case of real fish, the joint changes also occur quickly. In the fastest case we have observed, it took place in two days (data not shown), whereas the change from Fig. 2*b* to *d* took more than three months.

Although we do not have any information about the molecules which are involved in the pattern-forming reaction, it is possible to estimate roughly the diffusion coefficients of the molecules by comparing the simulation and the actual pattern changing of fish stripe. The stripe spacing is  $\sim 0.5$  cm in *P. imperator*, and  $\sim 10$  grids in the simulated patterns (Fig. 2); a grid in the simulation therefore represents 0.05 cm. The pattern change from Fig. 2*h* to *i* took 90 days (7,776,000 seconds) in reality, and 50,000 iterations in the simulation. The time step for the simulation therefore corresponds to 155.5 seconds. These values give diffusion coefficients of  $1.125 \times 10^7$  cm<sup>2</sup> s<sup>-1</sup> and  $1.608 \times 10^{-6}$  cm<sup>2</sup> s<sup>-1</sup> for the activator and the inhibitor, respectively. Both values are in the range of the diffusion coefficients of proteins in aqueous media<sup>9</sup>. However, the diffusive molecules may be smaller than proteins, because the diffusion rate of molecules is usually much smaller in real biological systems than in aqueous media.

In some other biological systems, the insertion of new structures during growth have been observed and simulated<sup>3,10-14</sup>. The novel features of the work reported here are that the inserted structure is a stripe and that the underlying mechanism is operative for a long period. The reaction-diffusion wave is a kind of standing wave. Therefore, to determine that a given pattern is consistent with a reaction-diffusion wave, it is necessary to impose some disturbance on the field and to see how the

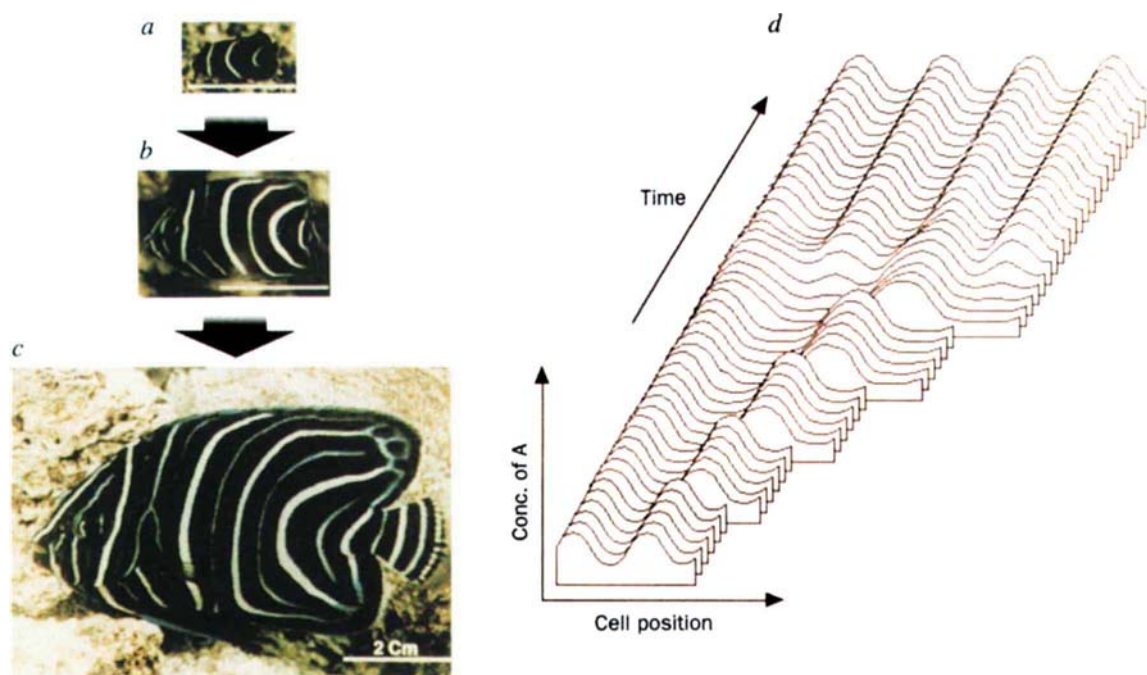


FIG. 1 Rearrangement of the stripe pattern of *Pomacanthus semicirculatus* and its computer simulation. *a-c*, Photographs of the juvenile of *P. semicirculatus*. Ages are  $\sim 2$  months (*a*),  $\sim 6$  months (*b*) and  $\sim 12$  months (*c*). Scale bars, 2 cm. *d*, Computer simulation of the reaction-diffusion wave on the growing one-dimensional array of cells. One of the five cells is forced to duplicate periodically (once in 100 iterations). Concentration of activator is represented as the vertical height. The equations for calculation are as follows:

$$\frac{dA}{dt} = c_1 A + c_2 I + c_3 - D_A \frac{d^2 A}{dx^2} - g_A A, \quad \frac{dI}{dt} = c_4 A + c_5 - D_I \frac{d^2 I}{dx^2} - g_I I$$

where *A* and *I* are the concentration of the activator molecule and the

inhibitor molecule, respectively,  $D_A$  and  $D_I$  are the diffusion constants,  $g_A$  and  $g_I$  are the decay constants, and  $D_A = 0.007$ ,  $D_I = 0.1$ ,  $g_A = 0.03$ ,  $g_I = 0.06$ ,  $c_1 = 0.08$ ,  $c_2 = -0.08$ ,  $c_3 = 0.05$ ,  $c_4 = 0.1$ ,  $c_5 = -0.15$ . Upper and lower limits for the synthesis rates of the activator ( $c_1 A + c_2 I + c_3$ ) and inhibitor ( $c_4 A + c_5$ ) are set as  $0 < c_1 A + c_2 I + c_3 < 0.18$  and  $0 < c_4 A + c_5 < 0.5$ . These upper and lower limits are set to avoid unrealistic situations. A moderate upper-limit value of the activator synthesis rate is required to get a pattern of stripes rather than spots<sup>15</sup> (spots are obtained if this value is exceeded). We used the kinetics of Turing<sup>1</sup>. Other stripe-forming interactions<sup>12,15</sup>, in which the upper and lower limit is a natural outcome of the kinetics, can simulate the fish pattern rearrangement reported here.

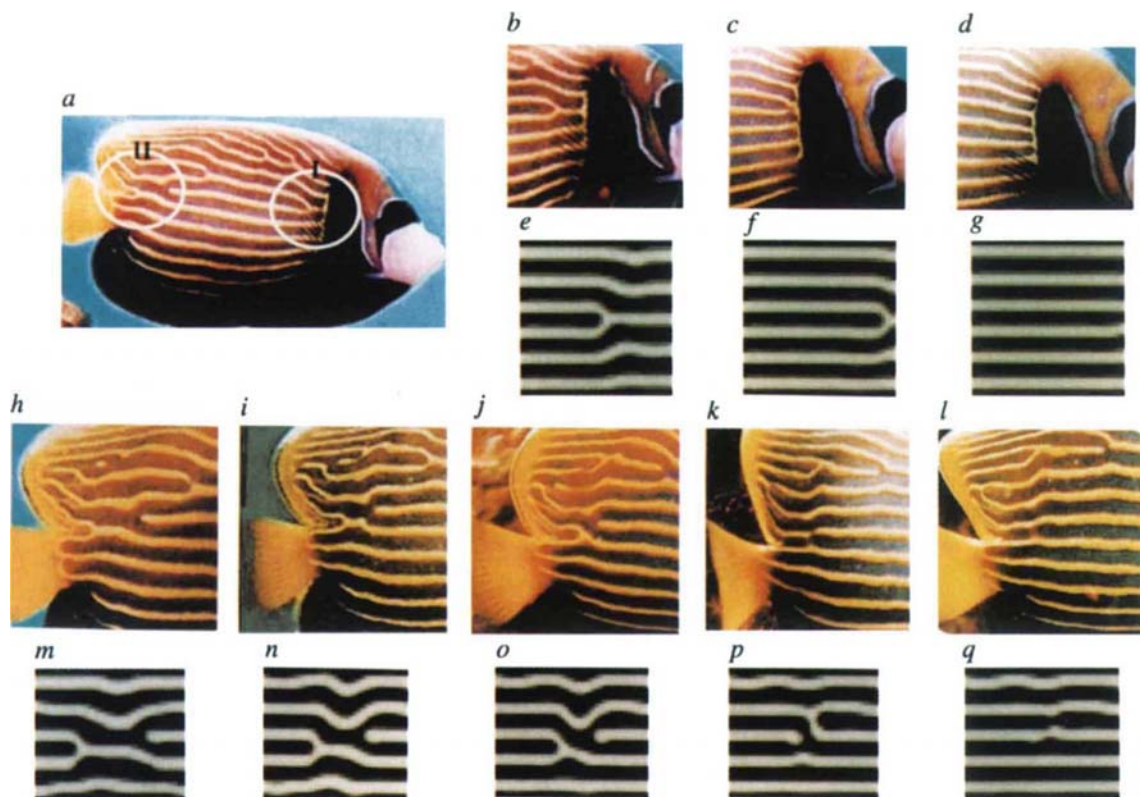


FIG. 2 Rearrangement of the stripe pattern of *Pomacanthus imperator* (horizontal movement of branching points) and its computer simulation. *a*, An adult *P. imperator* (~10 months old). *b*, Close-up of region I in *a*. *c*, *d*, Photographs of region I of the same fish taken two (*c*) and three (*d*) months later. *e*, Starting stripe conformation for the simulation (region I). *f*, *g*, Results of the calculation after 30,000 (*f*) and 50,000 (*g*) iterations. *h*, Close-up of region II in *a*. *i*–*l*, Photographs of region II of the same fish taken 30 (*i*), 50 (*j*), 75 (*k*) and 90 (*l*) days later, respectively.

*m*, Starting stripe conformation for the simulation (region II). *n*–*q*, Results of the calculation after 20,000 (*n*), 30,000 (*o*), 40,000 (*p*) and 50,000 (*q*) iterations, respectively. Fish (Fish World Co. Ltd (Osaka)) were maintained in artificial sea water (Martin Art, Senju). Skin patterns were recorded with a Canon video camera and printed by a Polaroid Slide Printer. In the simulated patterns, darker colour represents higher concentrations of the activator molecule. Equations and the values of the constants used, as Fig. 1.

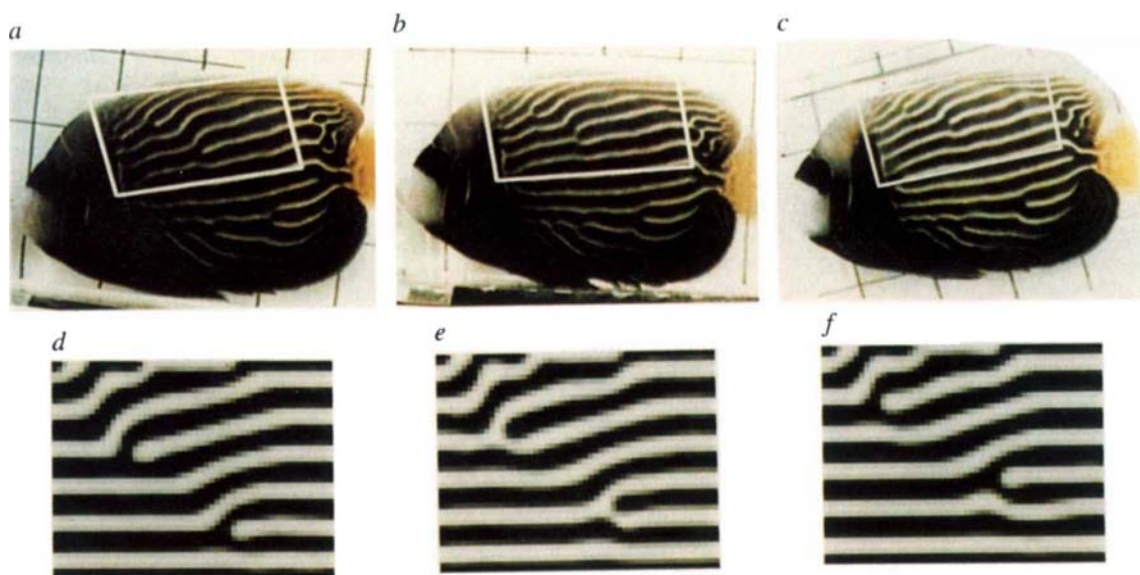


FIG. 3 Rearrangement of the stripe pattern of *P. imperator* (switch of joint) and its computer simulation. *a*, Photograph of a young *P. imperator* (~7 months old). *b*, *c*, Photographs of the same fish taken 6 (*b*) and 12 (*c*) days later. *d*, Starting stripe conformation for the simulation.

Pattern changing in the region surrounded by the white box was simulated. *e*, *f*, Results of the calculation after 2,000 (*e*) and 5,000 (*f*) iterations, respectively.

pattern responds. The pattern alteration of the *Pomacanthus*, accompanied by skin growth, can be taken as a natural experiment to help elucidate the underlying mechanisms which govern pattern formation. From the striking similarity between the actual and the simulated pattern alteration, it is highly probable that the mechanism is a reaction-diffusion system. Because the pattern-forming mechanism is maintained in adult skin, it should be possible to identify the molecules involved. □

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## Essential role for the *c-met* receptor in the migration of myogenic precursor cells into the limb bud

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**LIMB muscles develop from cells that migrate from the somites<sup>1,2</sup>. The signal that induces migration of myogenic precursor cells to the limb emanates from the mesenchyme of the limb bud<sup>2,3</sup>. Here we report that the *c-met*-encoded receptor tyrosine kinase is essential for migration of myogenic precursor cells into the limb anlage and for migration into diaphragm and tip of tongue. In *c-met* homozygous mutant ( $-/-$ ) mouse embryos, the limb bud and diaphragm are not colonized by myogenic precursor cells and, as a consequence, skeletal muscles of the limb and diaphragm do not form. In contrast, development of the axial skeletal muscles proceeds in the absence of *c-met* signalling. The specific ligand of the *c-met* protein, the motility and growth factor scatter factor/hepatocyte growth factor<sup>4–9</sup>, is expressed in limb mesenchyme and can thus provide the signal for migration which is received by *c-met*. We have therefore identified a paracrine signalling system that regulates migration of myogenic precursor cells.**

The *c-met*-encoded receptor tyrosine kinase and its ligand, scatter factor/hepatocyte growth factor (SF/HGF), regulate growth, motility and morphogenesis of cells *in vitro*<sup>4–10</sup>. A targeted mutation in the SF/HGF gene causes embryonic lethality and reveals essential roles of the factor in development of liver and placenta<sup>11,12</sup>. To investigate cell autonomous functions of

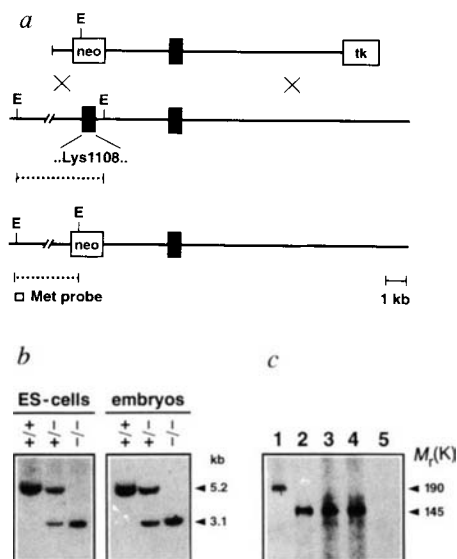


FIG. 1 Outline of the strategy to disrupt *c-met* and the effect of the mutation. **a**, Schematic representation of *c-met* targeting vector (top), wild-type *c-met* allele (middle) and mutant *c-met* allele (bottom). Exon sequences are represented by black boxes; neomycin resistance (*neo*) gene and thymidine kinase (*tk*) gene from herpes simplex<sup>25</sup> are shown and the triplet encoding the invariant Lys (amino acid 1,108; see ref. 26 for the numbering) is indicated. The open box indicates the probe used for Southern analysis (see **b**). Dashed lines correspond to *Eco*RI fragments detected in wild-type and mutant genomic DNA. **b**, Southern blot analysis of genomic DNA from wild-type (+/+) ES cells, from ES cells containing one (+/-) or two mutant (-/-) *c-met* alleles and from wild-type (+/+), heterozygous (+/-) and homozygous (-/-) mutant mouse embryos. **c**, *In vitro* auto-kinase assays on immunoprecipitated *c-met* protein from extracts of adult liver (*c-met* +/- animal) analysed under non-reducing (lane 1) or reducing conditions (lane 2). Auto-kinase activity of *c-met* protein immunoprecipitated from liver extracts of wild-type (+/+) (lane 3), heterozygous (+/-) (lane 4) or homozygous (-/-) embryos (lane 5) analysed under reducing conditions. In the absence or presence of reducing agents, *c-met* protein of 190 or 145K can be observed, that correspond to the  $\alpha$ - and  $\beta$ -chain linked by sulphhydryl bridges or to the separate  $\beta$ -chain, respectively. RNase protection analysis indicates that the mutant *met* allele produces normal levels of a  $\Delta$ -*met* transcript that lacks nucleotides 3,254–3,334 (data not shown). **METHODS.** Genomic *c-met* DNA isolated from a library of 129 mouse DNA (isogenic to ES cell line E14) was used to construct two targeting vectors. One exon (nucleotides 3,254–3,334 in the cDNA) of *c-met* was replaced by either wild-type or mutant<sup>27</sup> *neo* gene (*neo*<sup>wt</sup> or *neo*<sup>mu</sup>). The targeting vector containing *neo*<sup>mu</sup> was first introduced into E14-1 ES cells<sup>28</sup> by electroporation; homologous recombination events were enriched by selection with low G418 (0.2 mg ml<sup>-1</sup>) concentrations. Two independently mutated ES cell clones (met 30; met 34) were identified. The second targeting vector containing *neo*<sup>wt</sup> was electroporated into met 30 or met 34 ES cells; homologous recombination events were enriched by selection with high concentrations of G418 (1 mg ml<sup>-1</sup>). Three cell clones with two mutant *c-met* alleles (met 30-1; met 34-1; met 34-2) were identified. The structure of the mutant loci and absence of additional integration events was verified by Southern hybridization. Chimaera were produced by injections of mutant ES cells into C57BL/6 blastocysts (compare also Fig. 2). Mouse strains that carry the mutation were established from met 30 and met 34 ES cells. Phenotypes were analysed on C57BL/6/129 hybrid background. For protein analysis, extracts from embryonal or adult livers were precipitated with serum against a C-terminal *c-met* peptide produced essentially as described<sup>29</sup>. Immunoprecipitates were incubated for 30 min, 37 °C in 15  $\mu$ Ci of  $\gamma$ -<sup>32</sup>P-ATP (Amersham, 5,000 Ci mmol<sup>-1</sup>), 100 mM NaCl, 20 mM HEPES, pH 7.1 (*in vitro* kinase assay) and run on a SDS-polyacrylamide gel in the presence or absence of  $\beta$ -mercaptoethanol.

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this signalling system, embryonic stem (ES) cells that carry two mutant *c-met* alleles were generated. The targeting vectors used to mutate *c-met* were designed to replace 2 kilobases (kb) of genomic DNA that include the codon for an invariant lysine essential for kinase activity (Fig. 1a, b). Chimaeric animals were produced by injections into wild-type blastocysts and used to determine to what extent *c-met*<sup>-/-</sup> cells colonize different organs. ES and blastocyst-derived cells carry different alleles of glucose phosphate isomerase (GPI) that can be distinguished electrophoretically<sup>13</sup>. Determination of GPI isoenzyme ratios demonstrated that *c-met*<sup>-/-</sup> cells cannot contribute to skeletal muscles of the limbs, diaphragm and tip of the tongue. In addition, no contribution of *c-met*<sup>-/-</sup> cells to the liver was observed (Fig. 2). In contrast, *c-met*<sup>-/-</sup> cells were found to contribute to all other tested tissues including various skeletal muscles, such as body wall and axial muscles and other muscles of the head (Fig. 2). Several of the chimaeras were able to transmit the mutant gene through the germ line, indicating that *c-met* is not essential for male germ-cell development. Analysis of chimaeras obtained from injections of *c-met*<sup>+/-</sup> cells into blastocysts demonstrated that *c-met*<sup>+/-</sup> cells can contribute to all organs and cell types tested.

Mice strains carrying the *c-met* mutation were established. Animals with a heterozygous *c-met* mutation appeared healthy and were fertile. Breeding of heterozygous animals produced no homozygous mutant offspring, indicating that such animals die during development. Genotype analysis of embryos at different stages of gestation showed a mendelian ratio of viable *c-met*<sup>-/-</sup> embryos up to and including day 12.5 of embryogenesis (E12.5). The proportion of viable *c-met*<sup>-/-</sup> embryos declined to 16% on E14.5 and 2% on E16.5. No *c-met* autokinase activity was detected after immunoprecipitation of embryonic liver extracts from homozygous mutant embryos; *c-met* autokinase activity was present in control liver extracts (Fig. 1c). Thus, the introduced mutation interferes with production of functional receptor protein. On E14.5, the external appearance of *c-met*<sup>-/-</sup> embryos was normal except that a small reduction in size was observed compared to their wild-type or *c-met*<sup>+/-</sup> littermates; the reduction in size was further pronounced on E15.5 or E16.5, indicating that homozygous mutant embryos become progressively more delayed in development. Dissection of organs and histological analysis demonstrated a marked size reduction of the liver, damage of the liver parenchyma, and defects in placental development (data not shown). These abnormalities appear to be responsible for embryonic lethality and developmental retardation, and are identical to the defects observed in SF/HGF<sup>-/-</sup> mice<sup>11</sup>.

Myogenesis in limb buds, diaphragm and tip of the tongue appeared severely disturbed in *c-met*<sup>-/-</sup> embryos, in accordance with no contribution of *c-met*<sup>-/-</sup> cells to these particular muscles. When E15.5 embryos were analysed histologically, a complete absence of myotubes in the limbs, shoulders (Fig. 3a, b) and diaphragm was observed in *c-met*<sup>-/-</sup> embryos. A reduction in the overall size of the tongue was also noted, and detailed analysis revealed a reduction in the number of myotubes in the intrinsic tongue muscle, particularly at the tip of the tongue. In contrast, axial muscles (intercostal, ventral or dorsal body wall muscles) or other head muscles (extrinsic muscle of the tongue, masseter and ocular muscles) are formed in *c-met*<sup>-/-</sup> embryos. Similar defects in the formation of particular muscles can be observed in SF/HGF<sup>-/-</sup> embryos (F.B. and C.B., unpublished results). In *c-met*<sup>-/-</sup> E15.5 embryos, immunofluorescence analysis demonstrated an absence of muscle fibres or of myosin-expressing cells in the limbs (Fig. 3e; the limb of a control embryo Fig. 3c), shoulders, diaphragm (Fig. 3f; the diaphragm of a control embryo Fig. 3d) and a reduction in the number of cells that express myosin in the tongue (Fig. 3i; for comparison the tongue of a control embryo Fig. 3g). In *c-met*<sup>-/-</sup> embryos, myotubes are readily detected by myosin immunohistochemistry in intercostal (Fig. 3j; intercostal

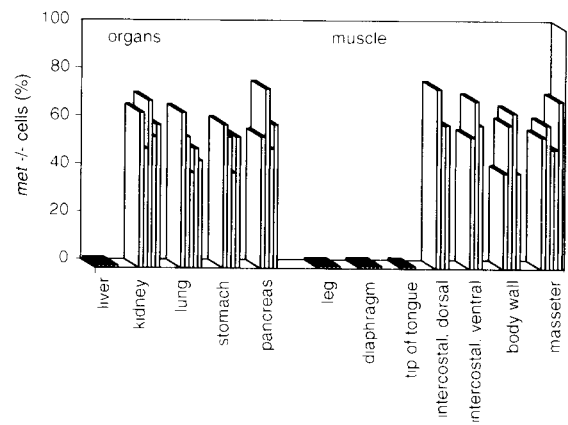


FIG. 2 Contribution of *c-met*<sup>-/-</sup> cells to various organs and cell types. Chimaeras were produced by injection of *c-met*<sup>-/-</sup> cells into wild-type blastocysts. Glucose phosphate isomerase isoenzyme assays were done to determine contribution of *c-met*<sup>-/-</sup> cells to various tissues and cell types of the adult chimaeras<sup>13</sup>. Displayed is the ES cell-derived contribution to indicated internal organs and muscles of individual animals. In addition, we tested thyroid gland, salivary gland, intestine, bladder, brain, eye, testis, ovary, thymus, spleen, spleen-derived B cells, T cells or macrophages, total blood cells and bone marrow. In all of these, an average *c-met*<sup>-/-</sup> cell contribution of 50% was observed. As a control, *c-met*<sup>+/-</sup> ES cells (clone 30 and 34) were injected into blastocysts; *c-met*<sup>+/-</sup> cells contributed effectively to liver, all muscles, organs and cell types tested.

**METHODS.** Two ES cell clones carrying one mutant *c-met* allele (ES cell clone 30; 34) and three cell clones that carry two mutant *c-met* alleles (ES cell clone 30-1; 34-1; 34-2) were used to generate chimaeric animals by blastocyst injections. The chimaerism of the animals was assessed by inspection of the coat colour (agouti versus black), and only highly chimaeric animals were used for further analysis. Different organs were dissected, total peripheral blood cells were isolated by centrifugation, spleen-derived B and T cells or macrophages were purified with Thy 1, Mac 1 or B220 monoclonal antibodies coupled to magnetic beads and MINIMACS columns (Miltrenyi Biotec Inc.). After separating GPI-1<sup>a</sup> and GPI-1<sup>b</sup> isoenzyme in tissue and cell extracts by electrophoresis (GPI-1<sup>a</sup> is expressed in 129 cells and thus in cells derived from ES cells; GPI-1<sup>b</sup> is expressed in C57BL/6 and thus in cells derived from blastocysts), the ES cell contribution was determined visually. Tissues or cell types from 3–5 individual animals obtained from injections of at least two independent ES cell clones were tested.

myotubes of a control embryo Fig. 3h), body wall or axial muscles and at other sites of muscle formation in accordance with the described expression patterns<sup>14</sup>.

We also analysed *c-met*<sup>-/-</sup> embryos for the expression of the myogenic determination factor myogenin, which encodes a helix-loop-helix transcriptional activator that is exclusively expressed in cells of the skeletal muscle lineage<sup>15</sup>. Myogenin-expressing cells were absent from the limbs (Fig. 3l; for comparison a control embryo in Fig. 3k), shoulders and diaphragm as tested by *in situ* hybridization on E10.5 or E12.5 embryos. Compared to wild-type or *c-met*<sup>+/-</sup> littermates, a marked reduction in the developing tongue was noted. Myogenin-expressing cells were readily detected in the limb and diaphragm of both *c-met*<sup>+/-</sup> and wild-type littermates in accordance with the described expression pattern<sup>15</sup>, or in developing axial (Fig. 3k, l) or head muscles of embryos, regardless of their genotype. Thus, the *c-met* mutation affects an early stage in the development of particular skeletal muscles.

Muscle precursor cells derive from dermo-myotome in the somites<sup>1,2</sup>. Ventro-lateral cells of the dermo-myotome can detach from the somites and can migrate to various sites in the embryo<sup>1,2,16</sup>. Particularly well studied is the migration of precursor cells into the limb, a site reached by myogenic cells between E9.5 and E10<sup>14</sup>. Pax-3 is expressed in dermo-myotome and in migrating myogenic cells and is therefore a useful marker



for identification of myogenic cells in the limb<sup>17,19</sup>. Pax-3-expressing cells neither migrate from the somites, nor are they detected in the limb or diaphragm of *c-met*<sup>-/-</sup> embryos between E9.5 or E12 (Fig. 4b), although such cells are apparent in the developing axial muscles. We found Pax-3 expression in the limb, and in other developing muscles of *c-met*<sup>+/-</sup> or wild-type littermates in accordance with the described temporal expression pattern<sup>17,18</sup> (Fig. 4a). Precursors for migratory myogenic cells in the lateral part of the dermo-myotome express Pax-3<sup>17-19</sup> and appear morphologically normal in *c-met*<sup>-/-</sup> embryos. We conclude therefore that in mutant embryos, muscle precursor cells are formed but do not migrate to the limb or diaphragm. As a consequence, muscles do not form at these sites.

The molecular nature of signals that induce migration of myogenic cells and direct the cells to their targets were previously unknown. If the *c-met* receptor receives such a signal, it should be expressed by myogenic precursor cells. Indeed, *c-met* is

expressed in the dermo-myotome, and particularly strong expression is observed at the ventro-lateral edge (Fig. 4c, e). In wild-type embryos, *c-met* expression is also detected in cells that migrate from the somites to the limb, and consequently, in cells that have reached the limb (Fig. 4c). In *c-met*<sup>-/-</sup> embryos (which express an altered *met* transcript,  $\Delta$ -*met*), the  $\Delta$ -*met*-expressing cells are not observed to migrate to or reach the limb bud. However, cells that express  $\Delta$ -*met* are present in the dermo-myotome (Fig. 4d) and the developing axial muscles. Interestingly, around the time of migration of myogenic cells (E9.5), mesenchymal cells in the limb are major sites of SF/HGF expression; the factor is expressed at high levels in the medial, but not lateral mesenchyme (Fig. 4f). Migration of myogenic precursor cells to the limb has been studied extensively, and essential signals were demonstrated to originate from the medial limb mesenchyme<sup>2,3</sup>. In contrast, the lateral mesenchyme does not produce this signal<sup>3</sup>. Thus, the effect of the mutation as well as the expression patterns are consistent with a role of *c-met* and SF/HGF as key regulatory molecules in the migration of myogenic cells. SF/HGF of the limb thus provides a spatially defined

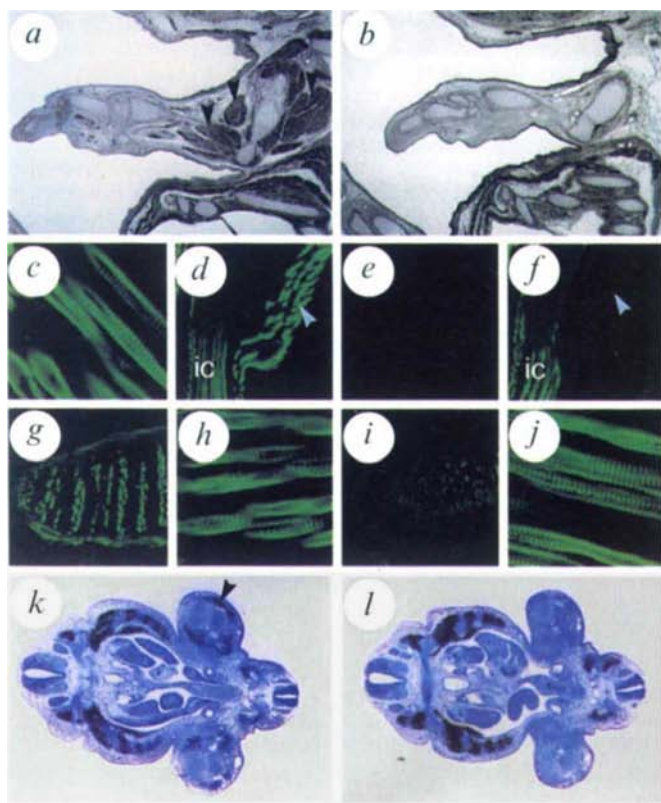


FIG. 3 Developing muscles in the limb and diaphragm are absent in *c-met*<sup>-/-</sup> embryos. Section of *c-met*<sup>+/-</sup> (a) and *c-met*<sup>-/-</sup> (b) embryos at E15.5, stained with desmin-specific antibodies and counterstained with haematoxylin. Limb muscles are indicated by arrows. Immunofluorescence analysis with anti-myosin-specific antibodies on E15.5 embryos: limb (c), diaphragm (d), tongue (g) and intercostal muscles (h) of *c-met*<sup>+/-</sup> embryos; limb (e), diaphragm (f), tongue (i) and intercostal muscle (j) of *c-met*<sup>-/-</sup> embryos. Arrows (d, f) point towards the diaphragm; intercostal muscles (ic) are indicated. *In situ* hybridization of *c-met*<sup>+/-</sup> and *c-met*<sup>-/-</sup> embryos on E12.5 with myogenin-specific probes (k, l). The arrows point towards myogenin-expressing cells in the limb bud of *c-met*<sup>+/-</sup> embryos (k); such cells are absent in the limbs of *c-met*<sup>-/-</sup> embryos (l).

**METHODS.** For haematoxylin and eosin staining, *c-met*<sup>+/-</sup> and *c-met*<sup>-/-</sup> embryos were embedded in Technovit 7100 (Heraeus Kulzer GmbH) and 8- $\mu$ m sections were used for staining. Immunohistochemistry was performed on sections of paraffin embedded embryos with monoclonal anti-myosin or anti-desmin IgG (Sigma) and FITC or peroxidase coupled anti-mouse IgG (Jackson Laboratories). For *in situ* hybridization, frozen sections were hybridized to <sup>35</sup>S-labelled myogenin RNA in antisense orientation.

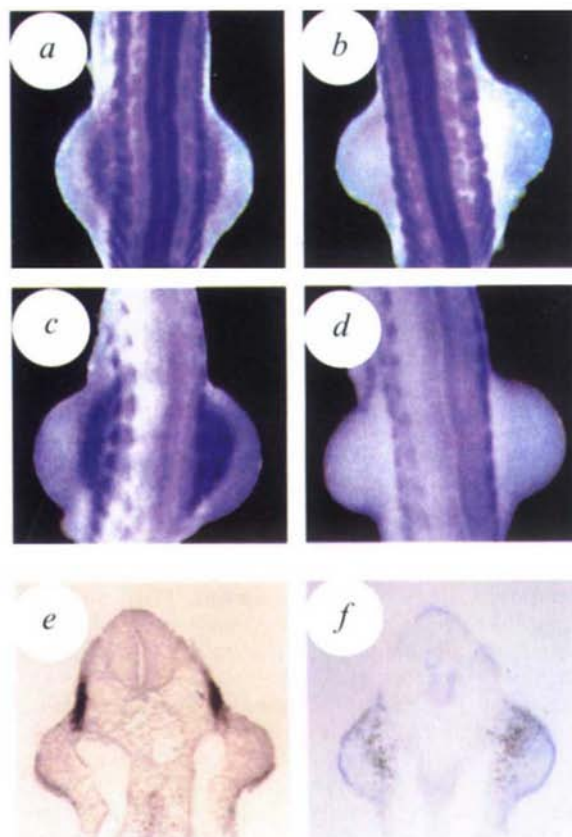


FIG. 4 Myogenic precursor cells do not migrate to the limbs in *c-met*<sup>-/-</sup> embryos. *In situ* hybridization analysis on *c-met*<sup>+/-</sup> (a, c) and *c-met*<sup>-/-</sup> (b, d) E9.5 embryos (27–28 somites), using Pax-3 (a, b) or *met* (c, d) specific hybridization probes. Note that *c-met*<sup>-/-</sup> embryos express a mutant *met* transcript ( $\Delta$ -*met*; see also Fig. 1). Pax-3 and *met* probes hybridize to myogenic precursor cells which are present in the limbs of *c-met*<sup>+/-</sup> embryos (a, c) but are lacking in the limbs of the *c-met*<sup>-/-</sup> embryo (b, d). Section of a wild-type embryo (E9.5, 27 somites), demonstrating a *c-met* hybridization signal in the ventro-lateral dermo-myotome and in cells that migrate from the dermo-myotome (e). *In situ* hybridization of a section from an embryo (E9.5, 27 somites) with a SF/HGF specific probe (f); SF/HGF is expressed in mesenchyme of medial limb at the time of myogenic cell migration.

**METHODS.** *In situ* hybridization on whole embryos or on frozen sections were performed with an antisense Pax-3<sup>17</sup>, *met*<sup>30</sup> or SF/HGF<sup>30</sup> RNAs. Corresponding sense transcripts did not produce distinct hybridization signals.

signal that induces the migration of myogenic precursor cells and could possibly play an additional role as a chemo-attractant for migrating cells, that is, direct them to the target.

The *c-met* receptor tyrosine kinase was first identified because of its oncogenic potential when mutated<sup>20</sup>. The specific ligand that binds to *c-met* and regulates the tyrosine kinase activity of the receptor is SF/HGF. The first activities described for this factor were the induction of motility of epithelial Madin Darby canine kidney cells<sup>5,7</sup> (scatter factor) and induction of growth of primary hepatocytes<sup>4</sup> (hepatocyte growth factor). *In vitro*, SF/HGF also induces invasion and migration in extracellular matrix<sup>21</sup>, a process reminiscent of cell migration *in vivo*. Other responses to SF/HGF include tubular morphogenesis<sup>22</sup> and not

only epithelial, but also endothelial and haematopoietic cells have been reported to respond to the factor<sup>23,24</sup>. Our genetic studies demonstrate a complex role for *c-met* in the control of growth, survival and migration of distinct embryonal cells. Processes that depend on SF/HGF and *c-met* require signal exchange between different cellular compartments that are located in close vicinity. The high affinity of SF/HGF for heparin, which is present on cell surfaces and extracellular matrix, might limit the diffusion and thus allow local actions of the factor only. The indistinguishable phenotypes we observe in mice with targeted mutations in SF/HGF or *c-met* also demonstrate that maternal SF/HGF can not diffuse and compensate for locally produced, embryonal factor, not even in the placenta. □

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## Mutation responsible for the mouse pygmy phenotype in the developmentally regulated factor HMGI-C

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GROWTH is one of the fundamental aspects in the development of an organism. Classical genetic studies have isolated four viable, spontaneous mouse mutants<sup>1</sup> disrupted in growth, leading to dwarfism. Pygmy is unique among these mutants because its phenotype cannot be explained by aberrations in the growth hormone–insulin-like growth factor endocrine pathway<sup>2–5</sup>. Here we show that the pygmy phenotype arises from the inactivation of *Hmgi-c* (ref. 6), a member of the *Hmgi* family<sup>7</sup> which function as architectural factors in the nuclear scaffold<sup>8</sup> and are critical in the assembly of stereospecific transcriptional complexes<sup>9</sup>. *Hmgi-c* and another *Hmgi* family member, *Hmgi(y)* (ref. 10), were found to be expressed predominantly during embryogenesis. The HMGI proteins are known to be regulated by cell cycle-dependent phosphorylation which alters their DNA binding affinity<sup>11</sup>. These results demonstrate the important role of HMGI proteins in mammalian growth and development.

The first step in the molecular definition of the pygmy mutation was made possible by the isolation of a transgenic insertional mouse mutant at the locus, *pg*<sup>TgN40ACha</sup> (ref. 12). A 0.5-kb *Apal*–*Apal* single-copy genomic sequence 2 kb from the site of

transgene insertion was identified<sup>12</sup> and used to initiate a bi-directional chromosome walk on normal mouse genomic DNA. The analysis of seven overlapping clones spanning 91 kb delineated a 56-kb common deletion between two informative mutants, *pg* and *pg*<sup>TgN40ACha</sup> (Fig. 1a).

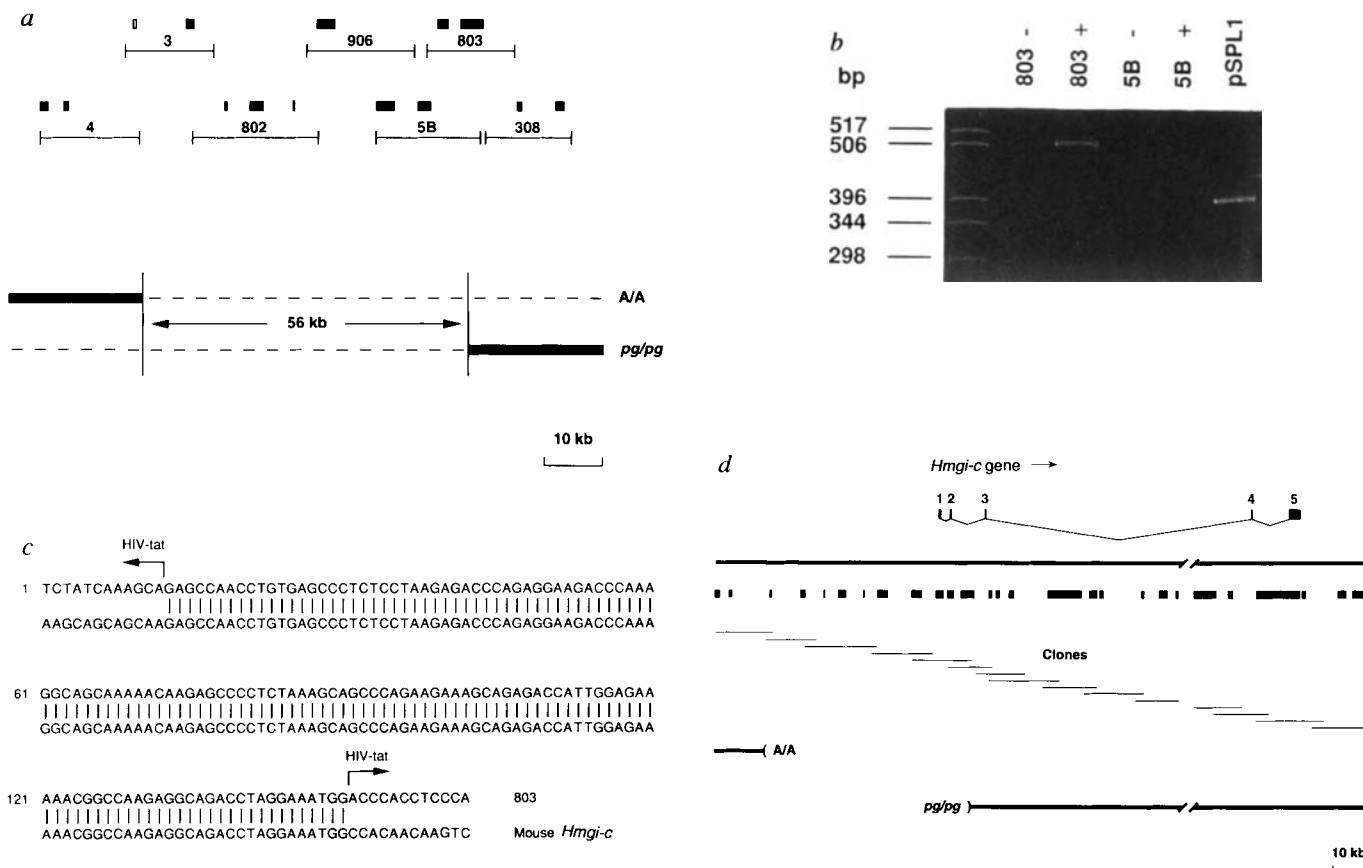
The common area of disruption was investigated further for candidate transcription units. The technique of exon amplification<sup>13</sup> was used to identify putative exons, and clones 803 and 5B, in the same orientation, produced spliced products (Fig. 1b). Their sequence was determined<sup>14</sup>, and a comparison with DNA sequence databases (GenBank and EMBL) revealed 100% homology to a previously identified gene, *Hmgi-c* (ref. 6) (Fig. 1c). *Hmgi-c* belongs to the *Hmgi* family of the general class of HMGI (high mobility group) DNA-binding proteins<sup>7</sup>. The HMGI proteins have been assigned multiple functions<sup>6</sup> and have been shown to be important in the regulation of gene expression as architectural factors by inducing DNA conformational changes in the formation of the three-dimensional transcription complex<sup>15,16</sup>.

The genomic structure of *Hmgi-c* revealed that it contains five exons and spans a region of approximately 110 kb (Fig. 1d). Single-copy sequences from the 190-kb cloned pygmy locus, surrounding and including the *Hmgi-c* gene (Fig. 1d), were used as probes on Southern blots containing DNA isolated from the two informative alleles<sup>12</sup>. The genomic area encompassing *Hmgi-c* is completely deleted in the transgenic insertional mutant *pg*<sup>TgN40ACha</sup> (A/A), whereas in the spontaneous mutant *pg* the 5' sequences and the first two exons are absent (Fig. 1d).

Previous studies<sup>17</sup> had established that the pygmy phenotype could be observed at birth. Therefore, RNA from wild-type mouse embryos was isolated<sup>18</sup>, and northern blot analysis revealed a transcript of 4.1 kb (Fig. 2). As expected from the genomic analysis, no detectable *Hmgi-c* expression was observed in the spontaneous and transgenic insertional mouse mutants. A third allele also exists at the pygmy locus<sup>1</sup>, *In(10)17Rk*,

which carries an inversion of chromosome 10, and the distal breakpoint is within intron 3 of the *Hmgi-c* gene (data not shown). No *Hmgi-c* expression was detected in homozygous embryonic *In(10)17Rk* RNA (Fig. 2). Quantification by phosphorimager analysis showed that heterozygous mice expressed *Hmgi-c* at approximately 50% of wild-type levels. Therefore the wild-type allele in the heterozygous mice does not increase

its expression levels to compensate for the loss of the deleted allele. This is consistent with the pygmy mutation being semi-dominant because there is a mild phenotypic effect on heterozygous mice (80% of the weight of wild-type mice)<sup>19</sup>. Furthermore, *Hmgi(y)*, the only other known member of the *Hmgi* gene family<sup>7</sup>, retained the same levels of expression in the mutant and wild-type mice (Fig. 2). Thus there is no



**FIG. 1** Identification and genomic characterization of the *Hmgi-c* gene at the pygmy locus in normal and mutant alleles. **a**, Delineation of the overlapping deleted genomic regions at the pygmy locus in the spontaneous and transgenic insertional mouse mutants. The open box above clone 3 positions the 0.5-kb *Apal*-*Apal* fragment, and the filled boxes represent single copy sequences used as probes to analyse genomic DNA isolated from mice of varying genotypes<sup>12</sup>. Solid and broken lines represent presence or absence of genomic sequences, respectively, in the transgenic insertional mouse mutant *pg*<sup>TGN40Acha</sup> (A) and the spontaneous mutant pygmy (pg). **b**, Exon amplification from lambda clones 803 and 5B. The primary polymerase chain reaction (PCR) exon amplification products in both sense (+) and antisense (-) orientations from the lambda clones shown in **a** were analysed on a 5% polyacrylamide gel<sup>13</sup>. The 379-bp PCR product observed in the control pSPL1 lane results from splicing between the HIV *tat* and  $\beta$ -globin vector sequences<sup>13</sup>. **c**, Sequence of exons amplified from clone 803 and comparison with the *Hmgi-c* gene. **d**, A series of overlapping phage clones extending approximately 190 kb at the pygmy locus. The discontinuous region represents an unclonable 11-kb fragment, as estimated from Southern blots of cleaved genomic DNA probed with single copy sequences from the end of the clonable region. The position and number of the *Hmgi-c* exons (not drawn to scale) are shown above the wild-type locus. Single copy sequences were isolated at the indicated positions and are represented by filled boxes below the wild-type locus. Thick bars and blank regions represent the genomic sequences that are present or deleted in the two alleles.

**METHODS.** The 0.5-kb *Apal*-*Apal* fragment<sup>12</sup> was used as a probe to isolate clones 3 and 4 from an EMBL3 mouse genomic library (a gift from E. Lacy) and a YAC (902C0711) from a mouse YAC library<sup>22</sup>. YAC 902C0711 was further subcloned into lambda FIX II (ref. 14), and 86

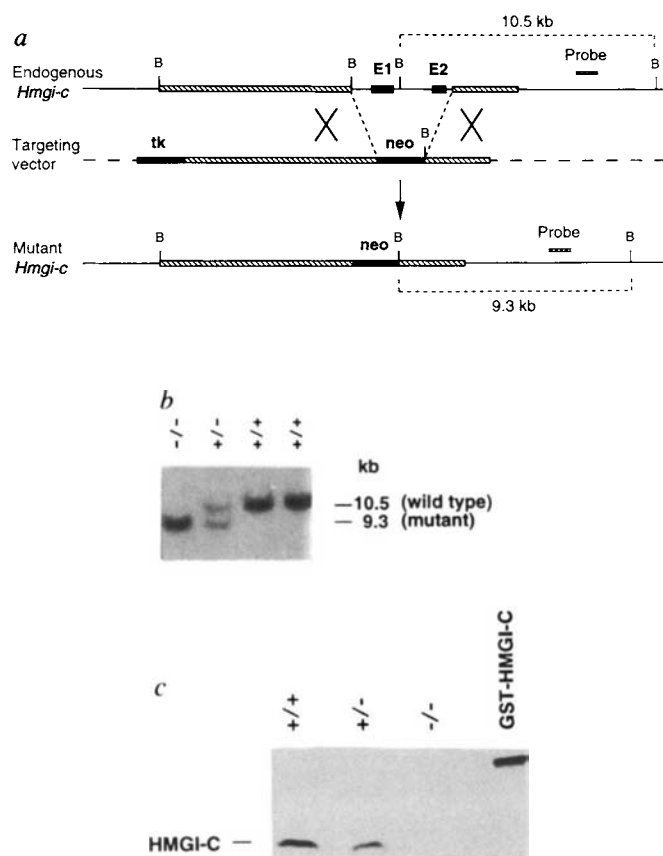
clones that hybridized to radioactively labelled mouse genomic DNA were picked and transferred to new plates in a grid array<sup>14</sup>. Lambda clones 802, 906, 5B, 803 and 308 were isolated after the walk was initiated with the 0.5-kb *Apal*-*Apal* fragment and accomplished by repeated hybridization to filters of the array. Overlaps between the contiguity clones and collinearity with the genome were confirmed by a combination of clone to clone and clone to genomic hybridizations and with restriction mapping. Exon amplification was done (Exon Trapping System, Gibco BRL) after the genomic inserts from the lambda clones were removed by cleavage with *Sall*, partly filled in<sup>14</sup> and subcloned into a partly filled-in *Bam*H1 cleaved pSPL1 plasmid<sup>13</sup>. The DNA was electroporated into COS-7 cells at 180 V and 960  $\mu$ F in a Bio-Rad Gene Pulser. Cytoplasmic RNA was isolated after 2-3 days and RT-PCR performed using primers supplied by the manufacturer. The secondary PCR amplification products<sup>13</sup> from clones 803 and 5B were subcloned into the plasmid vector, pAMP10 (Exon Trapping System, Gibco BRL) and sequenced using the Sequenase Version 2.0 sequencing kit (USB)<sup>14</sup>. A 344-bp fragment corresponding to the complete open reading frame of the *Hmgi-c* gene<sup>6</sup> was amplified from 12.5 d.p.c. mouse embryos (see text) using RT-PCR. Lambda clones containing the *Hmgi-c* exons were then isolated by hybridization of the 344-bp radioactively labelled fragment to the grid array of lambda clones and subsequently connected through chromosome walking. The RT-PCR conditions for isolation of the 344-bp fragment consisted of first strand cDNA synthesis with primer 1 (5'-ATGAATTCCTAATCCTCTCTGC-3'), followed by PCR amplification with primers 1 and 2 (5'-ATGATCCATGACGACGCGGT-3'). PCR conditions were 94 °C for 0.5 min, 55 °C for 0.5 min and 72 °C for 1 min, for 30 cycles. The amplified product was confirmed by sequencing analysis<sup>14</sup>.

FIG. 2 *Hmgi-c* gene expression of three alleles at the mouse pygmy locus. The wild-type allele is represented by +, the transgenic allele *pg*<sup>TgN40ACha</sup> by A, the spontaneous mutant allele by *pg*, and an allele at the pygmy locus which involves a paracentric inversion on chromosome 10 (*In(10)17Rk*) by Rk.

**METHODS.** The genotypes were established for mice in line A and the spontaneous mutant *pg* as previously described<sup>12</sup>, and mice containing the *In(10)17Rk* inversion were detected by a PCR-based restriction fragment length polymorphism (L. Cherath, K.F.B. and K.C., in preparation). RNA was isolated from 12.5 d.p.c. embryos, and equal amounts (5 µg) were analysed by northern blot hybridization<sup>14</sup>. The probes were a 138-bp nucleotide cDNA fragment encompassing exons 2 and 3 of the *Hmgi-c* gene, and a 340-bp cDNA fragment containing the complete coding sequence of the *Hmgi(y)* gene<sup>10</sup>. The blot was subsequently hybridized to an oligonucleotide complementary to murine 28S ribosomal RNA<sup>23</sup> to ensure that equal amounts of RNA were present in each lane, and the results are shown in the lower panel.

compensation by *Hmgi(y)* for the lack of *Hmgi-c* expression in pygmy mice.

The mutant alleles described above arise from major disruptions of genomic DNA which result in large deletions or a chromosomal inversion. To exclude the possibility that a gene other than *Hmgi-c* may be responsible for the pygmy phenotype, a mouse null mutant of *Hmgi-c* was produced by targeted disruption. Mouse embryonic stem (ES) cells were generated that had 3.0 kb of the *Hmgi-c* gene, encompassing exons 1 and 2, replaced with a neomycin-resistance gene (Fig. 3a). Matings between mice heterozygous for the mutated allele produced mice homozygous for the disrupted allele (Fig. 3b) at the expected mendelian frequency of approximately 25% (13 of 51). Immunoblot analysis demonstrated an absence of HMGI-C in protein extracts from homozygous embryos (Fig. 3c). Homozygous *Hmgi-c*<sup>-/-</sup> mice revealed the classical features of the pygmy phenotype, including reduced birth weight, craniofacial defects (shortened head), and an adult body weight of approximately 40%



( $39.8 \pm 2.9$ ) of that of wild-type littermates<sup>19</sup>. It can therefore be concluded that absence of *Hmgi-c* expression in mice results in the pygmy phenotype.

A restricted number of adult tissues had previously been analysed<sup>6</sup>, and it was established that the endogenous expression of *Hmgi-c* could not be detected. Hence, many more tissues were examined to investigate the temporal and tissue-specific expression pattern of *Hmgi-c*. Within the sensitivity of northern blot analysis, *Hmgi-c* expression was not detected in 18 adult tissues (data not shown). However, expression of *Hmgi-c* was observed during mouse embryogenesis (Fig. 4A) as early as 10.5 days post-coitum (d.p.c.), but essentially disappeared by 15.5 d.p.c. Remarkably, the other family member, *Hmgi(y)*, showed a similar endogenous expression pattern (Fig. 4A) with expression readily observed in 10.5–16.5 d.p.c. mouse embryos. The predominant expression of *Hmgi-c* and *Hmgi(y)* during embryogenesis suggests this architectural factor family functions mainly in mammalian development.

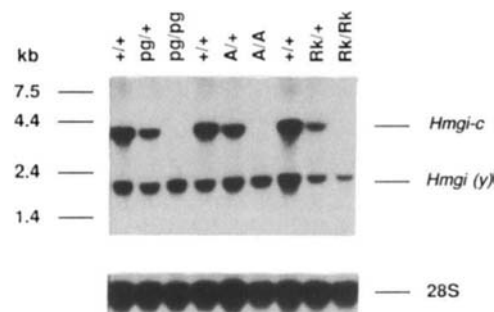
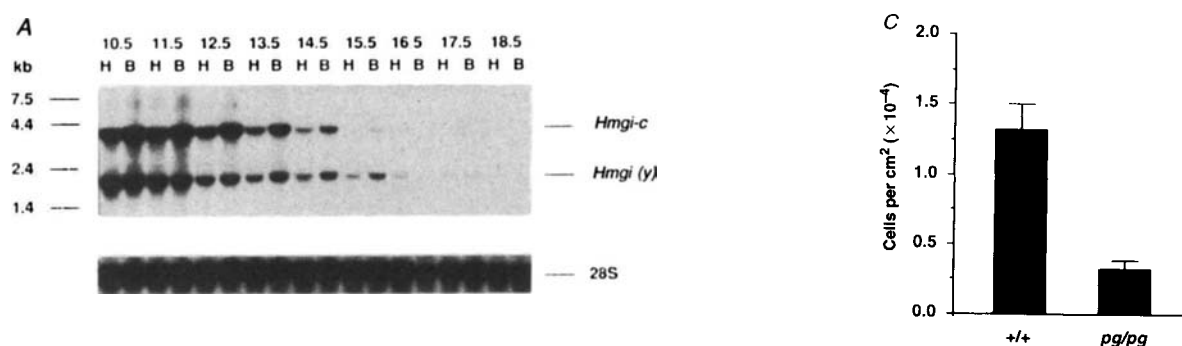


FIG. 3 Targeted disruption of the *Hmgi-c* gene. **a**, Targeting strategy. Endogenous *Hmgi-c* gene (top), targeting vector (middle) and predicted mutant gene (bottom). The targeting vector was created by replacing the 3-kb DNA fragment containing exon1 (E1) and exon2 (E2) with a PGK-neo cassette. The vector also includes a MC1-tk cassette at the 5' end of the long homologous segment. B, BamHI; Probe, a 4-kb *HincII* fragment used to identify the disrupted allele. **b**, Southern blot analysis of mice from a heterozygous cross. DNA from tails of the mice was digested with BamHI and hybridized to the external probe (see a). The positions of the bands corresponding to the wild-type allele (10.5 kb) and the mutant allele (9.3 kb) are indicated. **c**, Western blot analysis of wild-type (+/+), heterozygous (+/-) and homozygous (-/-) 12.5 d.p.c. embryos with anti-GST-HMGI-C rabbit IgG.

**METHODS.** Genomic clones of the mouse *Hmgi-c* gene were isolated from the mouse pygmy locus as in Fig. 1. Linearized vector (10 µg) was electroporated into AB1 ES cells at 280 V, 500 µF, and homologous recombination events enriched for by selection with G418 (350 µg ml<sup>-1</sup>) and 2 µM gangcyclovir (Syntex) on SNL76/7 feeder cells. Six targeted clones were obtained, and three were injected into C57BL/6J blastocysts to generate chimaeras. Chimaeric males were mated to C57BL/6J females, and heterozygous offspring intercrossed to produce subsequent generations. Southern blot analysis of the progeny from heterozygous crosses was performed as described<sup>14</sup>. Proteins were extracted from 12.5-d.p.c. mouse embryos from a heterozygous cross with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 10% glycerol, 5 mM magnesium acetate, 0.2 mM EDTA, 1.0 mM PMSF and 1% SDS. Each sample (10 µg) was separated by 15% SDS-PAGE, transferred to a nylon membrane (Duralon, Stratagene), and HMGI-C was detected using rabbit IgG anti-mouse GST-HMGI-C, HRP-conjugated goat anti-rabbit IgG, and ECL substrate (Amersham).





**FIG. 4** Expression of *Hmgi-c* in development and growth. **A**, Temporal expression pattern of *Hmgi-c* and *Hmgi (y)* determined by northern blot analysis of RNA (5  $\mu$ g) isolated from the head (H) and body (B) of mouse embryos whose ages (d.p.c.) are indicated. No expression of *Hmgi-c* was detected in placenta at any of these stages (data not shown). The probes are as in Fig. 2. **B**, Spatial localization of *Hmgi-c* transcripts in 11.5-d.p.c. mouse embryos. Photomicrographs of 8- $\mu$ m, adjacent, parasagittal sections through 11.5-d.p.c. mouse embryos hybridized with the antisense (a) or sense (b) strand of exon 2 and 3 of *Hmgi-c*, or stained histochemically with haematoxylin and eosin (c). G, gut mesenchyme; H, heart; L, Liver; Lb, limb bud; M, mandible; N, median nasal process; NE, neural epithelium; O, otocyst. Magnification,  $\times 15$ . **C**, Growth of wild-type and pygmy embryonic fibroblasts. Fibroblasts derived from 13.5-d.p.c. embryos were seeded at a concentration of  $1.7 \times 10^3$  cells per cm<sup>2</sup> in DMEM containing 10% fetal bovine serum. Cell number (ordinate) was determined on day 4. Small bars represent standard deviations of triplicate experiments;  $P < 0.001$ . The genotypes of embryos were determined as previously described<sup>12</sup>.

The analysis of *Hmgi-c* expression was further extended by its localization in the normal developing mouse embryo. Expression was observed in most tissues and organs during embryogenesis, as exemplified by the 11.5-d.p.c. mouse embryo shown in Fig. 4B. Noticeably, *Hmgi-c* expression was not seen in the embryonic brain except in a small, localized region of the fore-brain (Fig. 4B). This expression pattern coincides with previous studies which demonstrated that most tissues in pygmy mice were 40–50% smaller than wild-type tissues, the only tissue of normal size being the brain<sup>19</sup>.

To investigate the role of *Hmgi-c* in cell growth, embryonic fibroblasts were cultured from homozygous and wild-type embryos. The number of *pg/pg* embryonic fibroblasts was four-fold less than wildtype fibroblasts after four days *in vitro* (Fig. 4C), and was not due to cell death. These results, along with those from other systems<sup>20,21</sup>, are consistent with *Hmgi-c* being involved in cell proliferation and suggest that *Hmgi-c* functions

**METHODS.** For *in situ* hybridization, CBA/J embryos (11.5 d.p.c.) were fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Paraffin sections were deparaffinized and hybridized with sense and antisense riboprobes corresponding to exons 2 and 3 of *Hmgi-c* as previously described<sup>24</sup>. Sections were stained with haematoxylin and eosin according to standard procedures.

in a cell-autonomous manner. Furthermore, absence of *Hmgi-c* expression in the pygmy mutant would then lead to a decrease in cell proliferation and result in the reduced size of all the tissues except for the brain.

Our results demonstrate that the absence of *Hmgi-c* causes growth retardation in pygmy mice. Although the precise molecular mechanism is not yet known, the function of HMGI proteins in cell proliferation could be regulated during the cell cycle through alteration of their DNA binding ability through phosphorylation by the cell cycle-dependent p34<sup>cdc2</sup> kinase<sup>11</sup>. Inactivation of the *Hmgi-c* gene would perturb the cell cycle in the developing embryo, and the resulting disruption of growth would produce the pygmy phenotype. The identification of the pygmy gene as *Hmgi-c* provides an insight into the control of mammalian growth and development, and aids the investigation of the biochemical nature of the African pygmy phenotype<sup>4</sup> and a multitude of growth hormone-resistant human dwarf syndromes<sup>19</sup>. □

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# Familial Alzheimer's disease in kindreds with missense mutations in a gene on chromosome 1 related to the Alzheimer's disease type 3 gene

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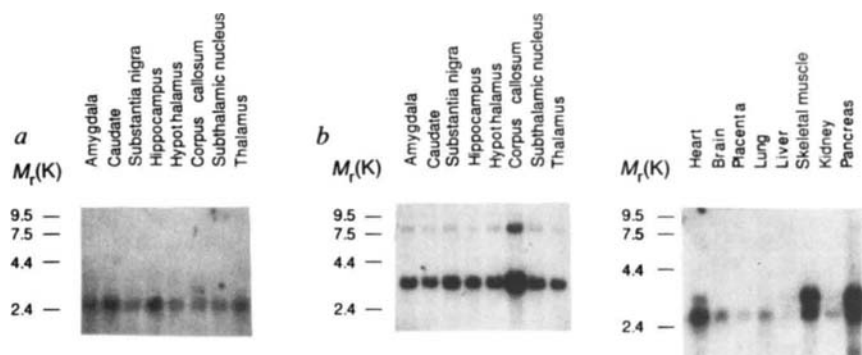
WE report the cloning of a novel gene (*E5-1*) encoded on chromosome 1 which has substantial nucleotide and amino-acid sequence similarity to the *S182* gene on chromosome 14q24.3. Mutations, including three new missense mutations in the *S182* gene, are associated with the *AD3* subtype of early-onset familial Alzheimer's disease (AD)<sup>1</sup>. Both the *E5-1* and the *S182* proteins are predicted to be integral membrane proteins with seven membrane-spanning domains, and a large exposed loop between the sixth and seventh transmembrane domains. Analysis of the nucleotide sequence of the open reading frame (ORF) of the *E5-1* gene led to the discovery of two missense substitutions at conserved amino-acid residues in affected members of pedigrees with a form of familial AD that has a later age of onset than the *AD3* subtype (50–70 years versus 30–60 years for *AD3*). These observations imply that the *E5-1* gene on chromosome 1 and the *S182* gene on chromosome 14q24.3 are members of a family of genes (*presenilins*) with related functions, and indicates that mutations in conserved residues of *E5-1* could also play a role in the genesis of AD. Our results also indicate that still other AD susceptibility genes exist.

Initial studies of the *S182* gene suggested the existence of other genes with similar sequences<sup>1</sup>. Searches of nucleotide sequence database by using the BLASTX alignment program uncovered three expressed sequence-tagged sequences (ESTs) (T03796, and subsequently R14600 and R05907) with substantial homology to two different segments within the *S182* ORF ( $P < 5.4 \times 10^{-11}$  identity >69% over at least 100 contiguous base pairs)<sup>2</sup>. Oligonucleotide primers were designed from these ESTs, and used to generate probes to screen complementary DNA libraries. Four cDNAs (*E5-1*, *G1-1*, *cc32* and *cc54*) were identified ranging in size from 1 to 2.3 kilobases (kb). The nucleotide sequence of these clones confirmed that all were derivatives of the same 2.3-kb transcript (designated *E5-1*). The gene encoding the *E5-1* transcript was mapped to human chromosome 1 by using hybrid mapping panels and the CEPH mega-YAC physical map (yeast artificial chromosome (YAC) clones 750g7, 921d12, 787g12) (results not shown).

The *E5-1* cDNA clones detected a low-intensity ~2.3-kb transcript on northern blots containing messenger RNA from several tissues, including most regions of the brain (Fig. 1). However, in skeletal muscle, cardiac muscle and pancreas, the *E5-1* gene is expressed at higher levels as two different transcripts of ~2.3 and ~2.6 kb (which we have not yet sequenced) (Fig. 1). Both of the *E5-1* transcripts have sizes clearly distinguishable from that of the 2.7-kb *S182* transcript, and did not cross-hybridize with *S182* probes at high stringency<sup>1</sup>.

The longest ORF within the *E5-1* cDNA consensus nucleotide sequence predicted a polypeptide containing 448 amino acids (Fig. 2). BLASTP alignment analyses detected significant homology with SPE-4 of *Caenorhabditis elegans* ( $P = 3.5 \times 10^{-26}$ ; identity = 20–63% over five domains of at least 22 residues), and weak homologies to several other transmembrane proteins similar to those previously reported for *S182* (ref. 1). However, the most striking alignment of *E5-1* was found with the amino-acid sequence predicted for the *S182* protein. These proteins share 63% overall amino-acid sequence identity, and several domains show almost complete identity (61–95% in the transmembrane domains) (Fig. 2). Furthermore, all of the residues mutated in *S182* in subjects with *AD3*, including three new mutations, are conserved in the putative *E5-1* protein (Fig. 2). As expected, hydrophobicity analyses indicate that both proteins could also share a similar structural organization, and are predicted to contain at least seven hydrophobic putative transmembrane domains, and large acidic hydrophilic domains at the amino terminus and between TM6 and TM7 (Fig. 3). An additional similarity between the *E5-1* and *S182* genes exists in the patterns of alternative splicing of the respective RNA transcripts. Analysis of cloned *E5-1* products of the polymerase chain reaction after reverse transcription (RT-PCR) isolated by using RNA from several tissues, including skeletal muscle and brain, revealed alternative splicing at nucleotides 1,153–1,250, which encode amino acids 263–296 in the TM6→TM7 loop of the *E5-1* protein (Figs 2 and 3). These residues share near-identity (31/33) with the alternatively spliced residues 257–290 in the TM6→TM7 loop of *S182* (which correspond to exon 9) (Figs

FIG. 1 Transcription of the *E5-1* homologue was investigated by hybridization of the *E5-1* cDNAs to northern blots of mRNA from human brain regions (a) and several peripheral tissues (c). The *E5-1* transcript is smaller and less abundant than the *S182* transcript, which is shown hybridized to the same blot using identical conditions (b)<sup>2</sup>.



2 and 3). In brain, the longer isoforms are the predominant transcripts for both *E5-1* and *S182*. However, whereas the shorter *E5-1* isoform can also be recovered from brain, the *S182* isoform lacking exon 9 is prominent only in leukocytes (manuscript in preparation).

The most noticeable differences between the two putative proteins occur in the central hydrophilic portion of the TM6→TM7 loop (residues 304–374 of *S182*; 310–355 of *E5-1*), and in the N terminus (Fig. 3). The TM6→TM7 loop domain is also less highly conserved between the murine and human *S182* genes (identity=47/60 residues), and shows no similarity to the equivalent region of SPE4 (Fig. 3 in ref. 1).

The strong overall similarity between *S182* and the *E5-1* gene products suggested that the *E5-1* gene might be the site of disease-causing mutations in a subset of AD pedigrees where linkage has been excluded from chromosomes 14, 19 and 21 (refs 3, 4). To test this, cDNAs corresponding to the *E5-1* transcript were amplified from lymphoblasts, fibroblasts or post-mortem brain tissue of affected members of 23 pedigrees with early-onset familial AD in which mutations in the *βAPP* and *S182* genes has previously been excluded by direct-sequencing studies. Examination of these RT-PCR products detected a heterozygous A→G substitution at nucleotide 1,080, which would cause a 239 Met→Val mutation in all four affected members of an extended pedigree of Italian origin (FLO10) with early-onset, pathologically confirmed familial AD (onset at 50 years) (Figs 2 and 4). A second heterozygous substitution (A→T at nucleotide 787) causing a 141 Asn→Ile missense mutation was found in affected probands of three out of four pedigrees of Volga German ancestry (represented by cell lines AG09369, AG09907, AG09952 and AG09905; Coriell Institute, Camden, New Jersey). The appearance of the same mutation in affected mem-

bers of three different Volga German pedigrees probably reflects a founder effect (additional cell lines from these families are not publicly available to prove intra-family segregation). The absence of the 141 Asn→Ile mutation in the fourth Volga German pedigree (AG09952) could be explained in two ways. The first is that the 141 Asn→Ile mutation is a benign polymorphism enriched in descendants of the original Volga German founders. The evolutionary preservation of the 141 Asn residue in the *S182/E5-1* family and the noticeably non-conservative nature of the Asn→Ile mutation argue against this. The alternative explanation is that subject AG09952 (who is aged 77 years) possesses another type of AD. This interpretation is supported by the fact that late-onset AD may affect at least 5% of the population older than 70 years (reviewed in ref. 5), and by the fact that while the Volga German populations are probably drawn from a limited number of founders, they are unlikely to represent a pure population isolate, particularly after emigration to North America in the last century<sup>6,7</sup>. This subject is also homozygous for the 4 allele of apolipoprotein E. Nevertheless, neither of the *E5-1* gene mutations was found in 284 normal Caucasian controls (Fig. 4), nor was either mutation present in affected members of pedigrees with the *AD3* type of AD. Moreover, both mutations would be predicted to cause substitution of residues that are highly conserved within the presenilin gene family (Fig. 2).

The discovery of a gene whose putative product shares substantial amino-acid and structural similarities with the *S182* gene product suggests that these genes may be functionally related. Further, the high degree of sequence identity in the conserved domains, and the clustering of mutations within those domains, argue that these domains may be critical either to correct intracellular targeting or more probably to a common function. The

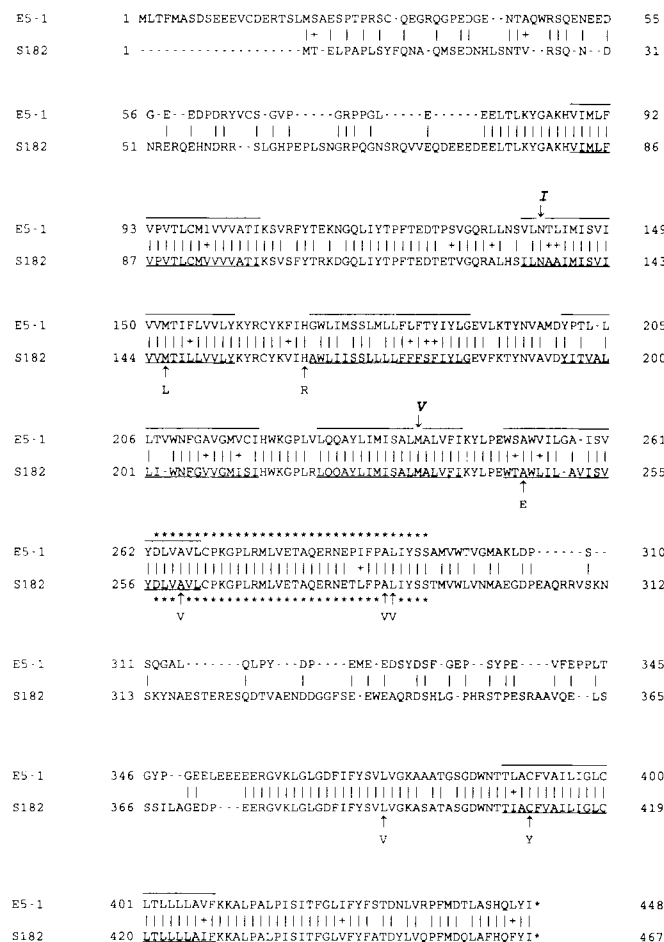
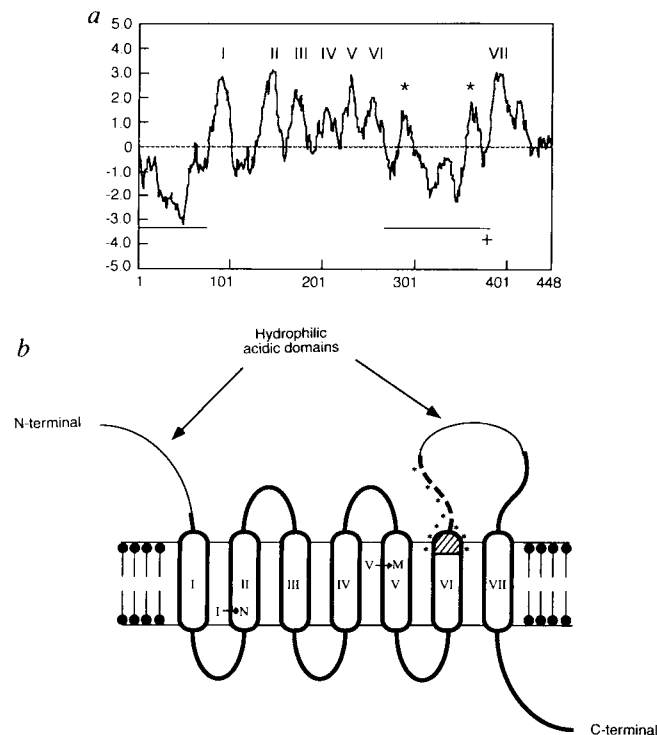
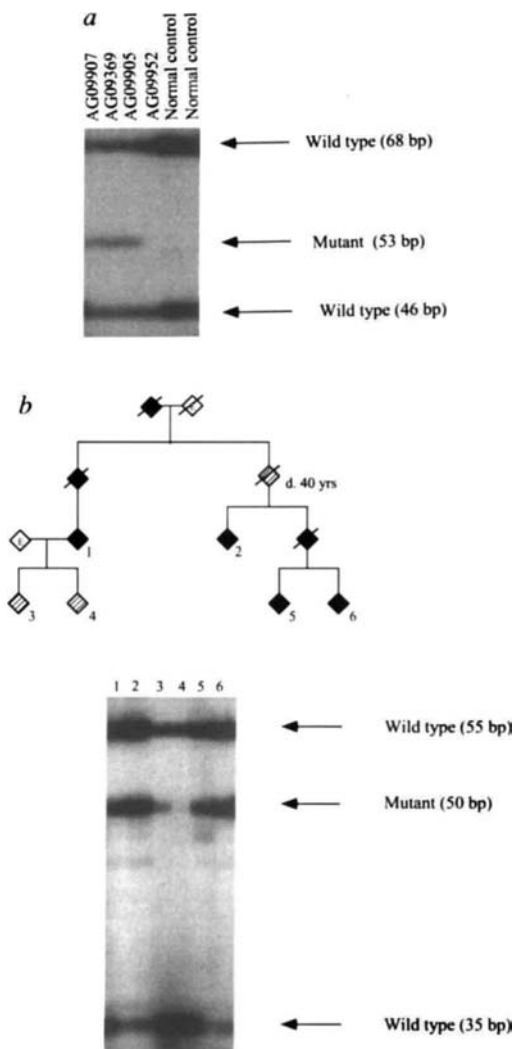


FIG. 2 Comparison of the predicted amino-acid sequence of the *E5-1* gene product (upper sequence) with that predicted for the *S182* gene product. Single-letter amino-acid codes are used, numbering from the first in-phase ATG codon which, for the *E5-1* gene, was surrounded by **GCC-agg-GCT-ATG**-c sequence which conformed to a translation start site. Identical residues are indicated by vertical lines, similar residues by +. The location of the putative mutations in the *E5-1* gene are indicated in bold type and by downward arrows above the *E5-1* sequence. The locations of the mutations in the *S182* gene are indicated by upward arrows below the *S182* sequence. Putative transmembrane (TM) domains are in open-ended boxes. The alternatively spliced exons are denoted by superscripted (*E5-1*) or subscripted (*S182*) asterisks. The nucleotide sequences have been deposited in the GSDB with accession numbers L42110 (*S182*) and L44557 (*E5-1*). The three new *S182* mutations were detected at 260 Ala→Val and 285 Ala→Val in two Japanese pedigrees, and at 392 Leu→Val in an Italian pedigree with early-onset familial AD (unpublished results). 260 Ala→Val and the 285 Ala→Val mutations: exon 9 was amplified from genomic DNA as previously described for the 286 Leu→Val mutation<sup>1</sup>. For the 260 Ala→Val mutation, end-labelled allele-specific oligonucleotides corresponding to the mutant sequence (994: 5'-GATTTAGTGGTTGTTTGTG) or the wild-type sequence (995: 5'-GATTAGTGGCTGTTTGTG) were hybridized at an annealing temperature of 48 °C, and washing was at 52 °C in 3 × SSC, 0.1% SDS. For the 285 Ala→Val mutation, allele-specific oligonucleotides for the wild-type sequence (1,003: 5'-TTTTCCAGTCTCATTTA) or the mutant primer (1,004: 5'-TTTTCCAGTTCTCATTTA) were hybridized at 48 °C and washed at 52 °C with 2 × SSC, 0.1% SDS. The Leu382Val mutation was scored by amplification of exon 13 from genomic DNA using oligonucleotides 996 (5'-AACTTGGATTGGGAGAT) and 893 (5'-GTGTGGCCAGGTAGAGAACT) with PCR as described previously for the L286V mutation<sup>1</sup>. End-labelled wild-type oligonucleotide (999: 5'-TACAGTGTCTGTTGTTGTTA) or mutant oligonucleotide (1,000: 5'-TACAGTGTCTGTTGTTGTTA) were hybridized at 45 °C and then successively washed in 2 × SSC at 23 °C and then at 68 °C.



**FIG. 3** *a*, Hydrophobicity plot for the putative E5-1 polypeptide using the hydrophobicity indices of Kyte and Doolittle with a calculation window of 15 residues<sup>9</sup>. The seven putative TM domains are numbered I-VII (top). The two apolar domains within the TM6→TM7 loop, which could be either TM domains or membrane-associated structural components of the TM6→TM7 loop, are denoted by asterisks. Hydrophilic domains are underlined. A single putative *N*-linked glycosylation site is indicated by a cross at residue 405. The Thr 281 in S182 is changed to Pro 287 in E5-1 which obliterates the first putative glycosylation sequence observed in S182. Comparison with the same plot for the putative S182 protein shows virtual overlap if allowances are made for the differences in the sizes of the acidic hydrophilic domains at the N terminus and between TM6 and TM7 (ref. 1). *b*, Predicted structure of the E5-1 polypeptide. Sequences conserved between E5-1 and S182 are depicted by thick lines, divergent sequences by thin lines. The location of the alternatively spliced domain is depicted by a broken line with asterisks. In the sorter isoform, the hatched space within TM6 would be removed at residue Asp 263 and spliced to residue Ser 296, thereby reconstituting TM6. The approximate locations of the mutations are noted by single-letter amino acid codes, with the wild-type sequence on the right.

**FIG. 4** Missense mutation in the E5-1 open reading frame: *a*, 141 Asn→Ile; *b*, 239 Met→Val. RT-PCR products corresponding to the E5-1 ORF were generated from RNA of lymphoblasts or frozen post-mortem brain tissue by using oligonucleotide primer pairs 1,021: 5'-CAGAGGATGGAGAGAATAC and 1,018: 5'-GGCTCCCCAAAACGTGCAT (product = 888 bp); and 1,017: 5'-GCCCTAGTGTTTCATCAAGTA and 1,022: 5'-AAAGCGGGAGCCAAAGTC (product = 826 bp) by PCR using 250 μmol dNTPs, 2.5 mM MgCl<sub>2</sub>, 20 pmol oligonucleotides in 50 μl, cycled for 40 cycles of 94 °C for 20 s, 58 °C for 20 s, 72 °C for 45 s. The PCR products were sequenced by automated cycle sequencing (ABI, Foster City, CA) and the fluorescent chromatograms were scanned for heterozygous nucleotide substitutions by direct inspection and by the Fatura (version 1.2.0) and Sequence Navigator (version 1.0.1b15) software packages (results not shown). Missense mutations were confirmed and their absence in non-AD control subjects was ascertained in genomic DNA as follows. 141 Asn→Ile: the A→T substitution at nucleotide 787 creates a *Bcl*I restriction site. The exon bearing this mutation was amplified from 100 ng of genomic DNA using 10 pmol of oligonucleotides 1,041: 5'-CATTCACTGAGGACACACC (end-labelled) and 1,042: 5'-TGTAGAGCACCACCAAGA (unlabelled), with PCR reaction conditions similar to those described for 239 Met→Val. 2 μl of the PCR product was restricted with *Bcl*I (NEBL, Beverly, MA) in 10 μl reaction volume according to the manufacturer's protocol, and the products were resolved by non-denaturing PAGE (polyacrylamide gel electrophoresis). With wild-type sequences, the 114-bp PCR product is cleaved into 68-bp and 46-bp fragments, whereas mutant sequences yield three products of 53, 46 and 15 bp. Subjects AG09907, AG09369, AG09905 and AG09952 (lanes 1-4) are AD-affected subjects of Volga German ancestry (Coriell Institute, Camden, NJ); the remaining subjects are asymptomatic caucasian controls 239 Met→Val: the A→G substitution at nucleotide 1080 deletes a *Nla*III restriction site. Amplification of 100 ng of genomic DNA with 10 pmol oligonucleotides 1,034: 5'-GCATGGTGTG-CATCCCACT, 1,035: 5'-GGACCACTCTGGGAGGTA: 0.5 U *Taq* polymerase, 250 μM dNTPs, 1 μCi [ $\alpha$ -<sup>32</sup>P]dCTP, 1.5 mM MgCl<sub>2</sub>, 10 μl volume; 30 cycles of 94 °C for 30 s, 58 °C for 20 s, 72 °C for 20 s, to generate a 110-bp product. 2 μl of the PCR reaction were diluted to 10 μl and restricted with 3 U of *Nla*III (NEBL) for 3 h. The restriction products were resolved by non-denaturing PAGE and visualized by autoradiography. Members of the FLO10 pedigree homozygous for wild-type sequences (products of 55, 35, 15 and 6 bp) or heterozygous for mutant sequences (products of 55, 50 and 6 bp) are shown.





nature of this function is unclear, but could reflect: (1) similar biochemical activities (perhaps with distinct substrate/ligand specificities); (2) different, perhaps consecutive, activities within the same biochemical pathway; or (3) subunits of a multimeric polypeptide like glutamate receptors<sup>8</sup>. The significant differences in the amino-acid sequences, and the occurrence of tissue-specific alternative splicing in the TM6→TM7 loop, suggest that this domain may be important, perhaps in determining gene-specific interactions.

A second conclusion derives from the observation of two different missense mutations in conserved domains of the *E5-1* protein in subjects with a familial form of AD. The evidence associating mutations in *E5-1* to the cause of AD in some pedigrees is less rigorous than the link between similar mutations in *S182* and early-onset AD. Nevertheless, if these observations could be confirmed by the analysis of additional pedigrees, it would strengthen our hypothesis that the *S182* and *E5-1* gene products subserve the same biochemical pathway, and that perturbations in that pathway augment the risk for acquiring AD. Although the disease phenotypes in the pedigrees with *S182* mutations are generally similar to those with mutations in *E5-1*, there are subtle differences (*S182*, onset at age 30–50 years, duration 10 years; *E5-1*, onset at age 50–70 years; duration up to 20 years) (refs 6, 7 and S.S., unpublished results). These differences could be accounted for by the lower level of expres-

sion of the *E5-1* transcript in the central nervous system, by differences in tissue-specific alternative splicing of *E5-1*, or by a different specific activity of the *E5-1* gene product.

Finally, although our data indicate that still other AD-susceptibility genes exist, knowledge of the biochemical functions of both of these genes will probably be helpful in unravelling the processes that cause AD. □

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## Modulation of conscious experience by peripheral sensory stimuli

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**LACK of awareness of touch associated with brain damage may transiently recover after stimulation of the vestibular system<sup>1,2</sup>. We used positron emission tomographic regional cerebral blood flow measurements to study the neurophysiological effect of vestibular stimulation on touch imperception in a subject with a right brain lesion. We tested the hypothesis that the vestibular system aids conscious tactile perception by introducing a bias in the neural system subserving body representation. We show that in normal subjects touch and vestibular signals share projections to the putamen, insula, somatosensory area II, premotor cortex and supra-marginal gyrus. In our patient a subset of these regions (right putamen and insula) was spared by the lesion and was maximally active when touch and vestibular stimulations were combined. These results support the suggestion that our phenomenological consciousness is associated with activation in circumscribed brain areas specific to the particular sensation of which we are aware.**

In this experiment we were interested in studying how perceptual awareness can be modulated by peripheral stimulation. Our model capitalizes on the observation that touch imperception caused by cerebral damage may transiently recover after appropriate caloric stimulation of the vestibular system<sup>1,2</sup>. The modulatory effect of caloric vestibular stimulation on touch imperception suggests that vestibular and touch projections may, to some extent, overlap in the brain. We have explored this hypothesis in four normal adult right-handed males who underwent 12 positron emission tomographic (PET) measurements of regional cerebral blood flow (rCBF), an index of synaptic activity<sup>3,4</sup>.

Two subjects received light, short tactile stimuli of the left hand during each of 6 of their 12 scans. Tactile stimulation was delivered by the examiner with the tip of the index finger every 2 s during PET scans. Subjects explicitly confirmed after each scan that they had consistently perceived the tactile stimuli. The area stimulated was the centre of the fourth metacarpal space of the left hand because this stimulus was previously used in an experiment showing transient remission of tactile imperception following cold vestibular stimulation (cold water was poured in the outer ear) in brain-damaged patients<sup>1</sup>. The other two subjects had left caloric vestibular stimulation before 6 of their 12 scans. Left vestibular stimulation with cold water was delivered for 30 s and stopped 10 s before the beginning of scanning, which coincided with arrival of radioactivity in the head. Therefore, tactile components of caloric stimulation did not contaminate the scan signal as the method is blind to brain activity occurring before the arrival of blood flow tracer in the brain<sup>4</sup>. The scans thus captured brain activity caused by vestibular symptoms only, which come on after a short delay from starting caloric stimulation and persist for a minute or so after it ceases<sup>5</sup>. After scan completion we confirmed that subjects showed the typical tonic deviation of gaze towards the side of cold stimulation and elicited explicit reports of transient dizziness and vertigo. rCBF was also measured six times during an eyes-closed resting state (baseline condition) in all four subjects. The order of the experimental and control scans was counterbalanced in both experiments to avoid habituation effects.

Anatomical overlap between touch and vestibular pathways

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TABLE 1 Stereotactic coordinates and statistical magnitude of brain areas

| (a) Activated in normal controls by both tactile stimulation of the left hand and left cold vestibular stimulation |     |     |    |         |
|--------------------------------------------------------------------------------------------------------------------|-----|-----|----|---------|
|                                                                                                                    | x   | y   | z  | Z score |
| Putamen (right)                                                                                                    | 20  | 2   | -4 | 3.7     |
| Insula (right)                                                                                                     | 34  | 4   | 4  | 6.1     |
| Insula (right)                                                                                                     | 28  | -4  | 16 | 4.0     |
| Thalamus (left)                                                                                                    | -16 | -16 | 12 | 3.8     |
| Retroinsular cortex-SII (right)                                                                                    | 26  | -26 | 16 | 4.4     |
| PMC (left)                                                                                                         | -48 | -2  | 8  | 4.0     |
| PMC (right)                                                                                                        | 42  | 4   | 16 | 4.2     |
| SMG (right)                                                                                                        | 42  | -32 | 24 | 4.5     |
| (b) Significant interaction between touch and vestibular stimulation in patient R.F.                               |     |     |    |         |
|                                                                                                                    | x   | y   | z  | Z score |
| IFG (right)                                                                                                        | 36  | 32  | 0  | 2.6     |
| Insula (right)                                                                                                     | 36  | 0   | 12 | 2.6     |
| Putamen (right)                                                                                                    | 22  | -2  | -4 | 1.8     |

PMC, Premotor cortex; SMG, supramarginal gyrus; SII, secondary sensory area; IFG, inferior frontal gyrus.

was assessed as the combined (main) effect of the experimental procedures compared to baseline. rCBF PET data were analysed with Statistical Parametric Mapping (SPM—Wellcome Department of Cognitive Neurology, London)<sup>6,7</sup> using the *t*-statistic (see Fig. 1 legend). Table 1a reports stereotactic coordinates of significant rCBF increases during both touch and vestibular stimulation (statistical maps are shown in Fig. 1). Significant activation foci were detected in the right putamen, insula, premotor cortex, somatosensory area II and inferior parietal cortex. We propose, therefore, that these brain regions constitute conjoint projection areas of the somatosensory and vestibular afferent pathways.

We then studied the neurophysiological bases of the interaction between vestibular stimulation and awareness for tactile stimulation in a patient with left tactile imperception. R.F. was a right-handed, 65-year-old man who suffered a right hemispheric stroke. At onset he showed left tactile imperception (hemianaesthesia) and a hemiparesis. Four weeks after the stroke, R.F. was densely hemianaesthetic without signs of unilateral spatial neglect. Awareness for tactile stimulation recovered transiently, for about 30 min, after left cold vestibular stimulation<sup>1,2</sup> (Fig. 2a). Computed tomography and averaged rCBF scans showed a cerebral lesion which involves a large portion of the right primary somatosensory and motor areas, supramarginal gyrus, and possibly somatosensory area II (Fig. 3). Alternating rest and touch PET scans were performed as in the normal subjects. However, from scans 4 to 9, preceding left cold vestibular stimuli were administered, resulting in recovery of conscious tactile perception during scanning (Fig. 2b, c). Scans 10–12 were performed when conscious touch perception had disappeared and the patient was again unaware of being touched. To examine the neurophysiological basis of this recovery of conscious touch perception we looked for positive interactions between touch and vestibular stimulation manifesting in the brain as modulations of touch-induced regional activity associated with concurrent caloric vestibular stimulation. Conscious compared with unconscious touch process (conscious touch versus rest with caloric stimuli)–(touch imperception versus rest without caloric stimuli) was associated with maximal increases of activity in right putamen and insula. Stereotactic anatomical locations of these foci were almost identical to a subset of those observed to receive vestibular and touch afferents conjointly in normal subjects. We also found a positive interaction in the right basal prefrontal cortex that did not correspond to any of the joint somatosensory–vestibular projections (Table 1b, Fig. 3).

Normal subjects show transient imbalance of their egocentric body coordinates after cold vestibular stimuli: objective

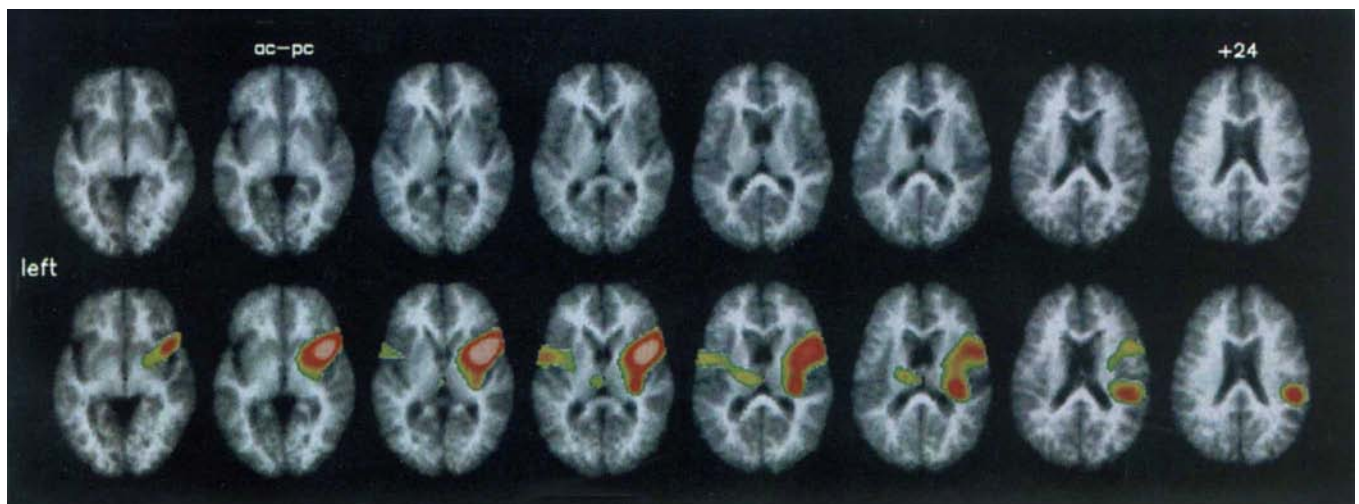
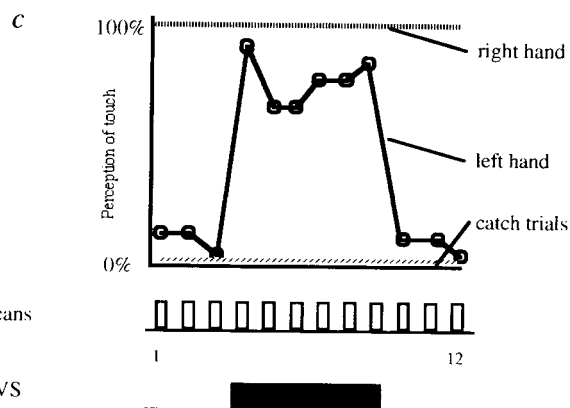
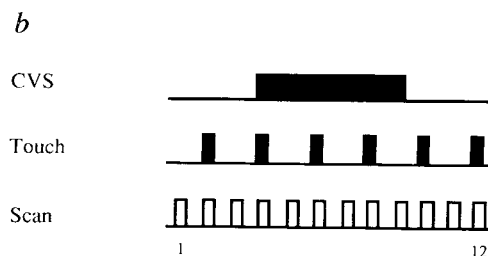
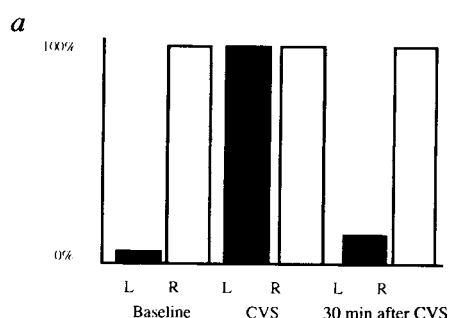


FIG. 1 Areas of overlap between touch and vestibular projections. The top row illustrates average magnetic resonance imaging (MRI) maps from all normal subjects in transaxial slices in stereotactic anatomical space<sup>12</sup>. The locations of rCBF increases associated with both touch stimulation and vestibular stimulation are shown as *t*-maps in the second row, overlapped on the same MRI template. Numbers refer to the distance in millimetres of the transaxial images from the intercommissural (ac–pc) plane. The exact coordinates of the activated areas and their significance levels are given in Table 1a.

**METHODS.** rCBF was measured by recording the distribution of radioactivity following the intravenous injection of <sup>15</sup>O-labelled water (H<sub>2</sub><sup>15</sup>O) with the CTI 953B PET scanner (CTI Inc., Knoxville, TN, USA). Data were acquired by scanning in 3-D mode<sup>13</sup>. A 'slow' bolus of H<sub>2</sub><sup>15</sup>O was infused as a tracer of blood flow while scans were acquired<sup>4</sup>. After attenuation correction (measured by a transmission scan), the data were recon-

structed as 31 transaxial planes by three-dimensional filtered back projection with a Hanning filter of cut-off frequency 0.5 cycles per pixel. The resolution of the resulting images was 8.5 × 8.5 × 6.0 mm at full-width half-maximum<sup>14</sup>. The integrated counts accumulated over 90-s scans were used as an index of rCBF<sup>15</sup>. The original brain PET images were transformed into standard stereotactic anatomical space<sup>12,16</sup>. MRI images from each individual were transformed in the same way to generate an optimal template for accurate anatomical identification of activation foci. By using appropriate linear contrasts, a combined (main) effect of touch and vestibular stimulation was estimated on a voxel by voxel basis using Statistical Parametric Mapping (SPM). Global differences in CBF were first covaried out<sup>6</sup>, and comparisons of the means were made using the *t*-static<sup>7</sup> (*P* < 0.05, corrected for multiple comparisons). The resulting set of *t* values constituted a statistical parametric map (SPM{*t*}<sup>7</sup>).



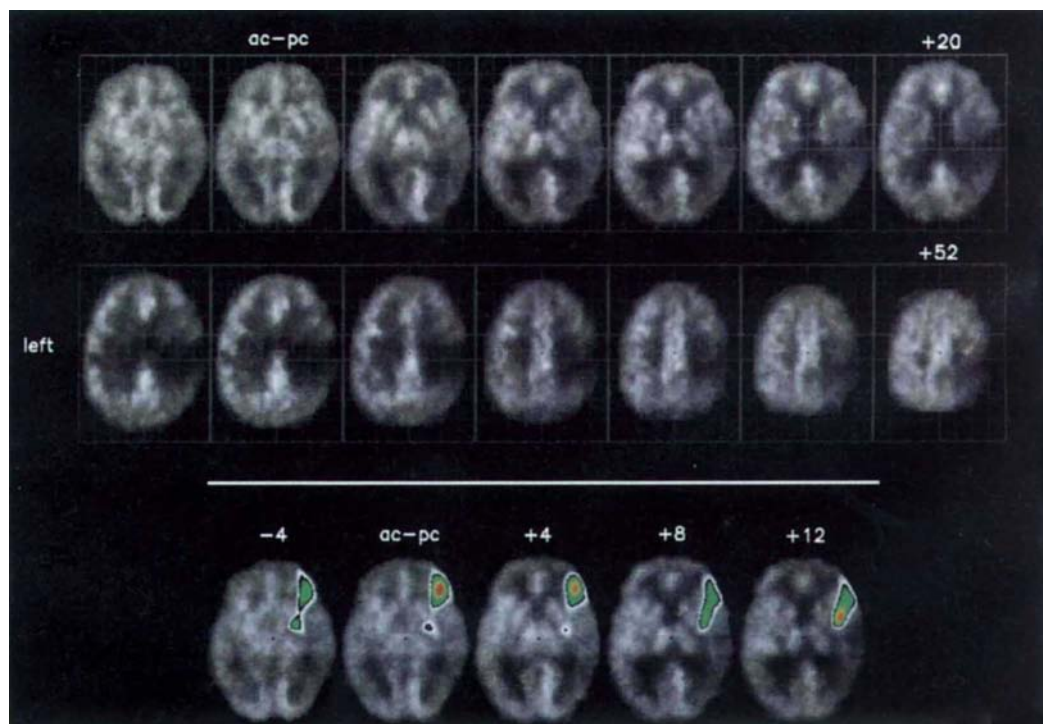
**FIG. 2** Behavioural profile of patient R.F. before PET scanning, scanning procedure and behavioural profile during the PET experiment. *a*, Thirty light, short, tactile stimuli were delivered by the examiner using an index finger, in the fourth metacarpal space of the left or right hand, in a randomized order, while the patient had his eyes closed. Each stimulus was preceded by a verbal warning. The patient was instructed to answer 'yes' if he felt a touch and 'no' if he did not. To control the reliability of affirmative responses 10 randomized catch trials were introduced (not shown in the figure). A dense left hemianaesthesia was found and then left cold vestibular stimulation was administered. No false alarms were recorded in the catch trials. After stimulation, leftward tonic deviation of gaze was observed. Remission of left touch imperception lasted 30 min. Then the patient returned to the hemianaesthetic state. The same examination was repeated after a 24 h delay on two occasions with identical results. *b*, Subject R.F. underwent 12 rCBF PET scans. During scans 2, 4, 6, 8, 10 and 12, light, short, tactile stimuli were delivered by the examiner on the left hand, at a rate of one stimulus every 2 s. Left cold caloric vestibular stimulation (CVS) was delivered from scan 4 to scan 9 with the same procedure used for normal subjects (see the main text). *c*, The effect of left cold CVS on touch perception was assessed during the interval between scans. The figure shows the percentage of touch perception in both hands and performance on catch trial. A dramatic recovery from left hemianaesthesia was

observed following CVS. Perception in the right hand was always at ceiling. Scan 10 was performed only after touch perception returned to baseline.

**FIG. 3** Functional lesion in patient R.F. and brain areas of positive interaction between touch and vestibular stimulation. The first two rows show averaged rCBF maps from subject R.F. transformed into a standard stereotactic space. Abnormal asymmetry of rCBF is evident (from plane +8 mm to plane +52 mm from the ac–pc plane) in putative somatosensory area II, primary somatosensory area and supramarginal gyrus. The functional lesion was due to ischaemic stroke involving the same areas on CT scan. The third row illustrates the brain areas where a positive interaction between touch and caloric vestibular stimulation (CVS) on rCBF was found.

**METHODS.** Positive interaction between touch and CVS on rCBF were formally assessed. The experiment on subject R.F. was treated as  $2 \times 2$  factorial design (touch: + and –; CVS: + and –).

Brain areas where CVS and touch interact were identified as positive interactions between the two factors. These areas were also required to have maximal rCBF when the subject was aware of being touched. Global differences in CBF across tasks were first covaried out<sup>6</sup>, and



comparisons of the means were made using the *t* statistic<sup>6,7</sup>. As the brain areas of CVS–touch interaction were predicted on the basis of the conjoint activation pattern shown by normal volunteers, a statistical threshold of  $P < 0.05$  (non-corrected) was applied to the SPM $\{t\}$  maps<sup>7</sup>.

measurements of such disorientation can be made by monitoring systematic directional bias when people point towards the centre of space with their eyes closed<sup>8</sup>. Such spatial bias is associated with strongly asymmetrical activation of central vestibular projection areas<sup>8</sup>. Unilateral brain damage produces long-term asymmetry of activity in those brain areas into which the vestibular and touch systems share afferent projections. Accordingly, touch imperception can be viewed as the result of a distorted body representation generated by a distributed neural system whose activity is set at a low level in anatomically spared regions and at zero in those areas directly damaged (Fig. 3). Recovery of touch imperception suggests that sensory body representations must exist in the areas that we have identified. There is evidence, for example, that tactile signals arrive in the insula<sup>9,10</sup>. An undamaged subset of these representations, retaining sub-cortical afferents, is able to mediate crude tactile perception when an appropriate physiological bias or modulation is introduced that has sufficient anatomical specificity to reduce the representational distortion produced by the lesion. The novelty in our observations is that we have associated recovery of the conscious awareness of touch with modulatory activity generated by vestibular stimulation in a set of touch areas that normally also receive vestibular stimuli. Our experiment clearly shows that primary somatosensory cortex has no primacy for conscious tactile perception, rather, multiple sensory representations of the body

exist in the brain and all of these can contribute to perception. This is more evidence for the contention of ref. 11 that '... each (cortical) area is capable of contributing directly and explicitly to ... perception in relation to its capacities ...'. □

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## Receptor-tyrosine-kinase- and G $\beta\gamma$ -mediated MAP kinase activation by a common signalling pathway

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MITOGEN-ACTIVATED protein (MAP) kinases mediate the phosphorylation and activation of nuclear transcription factors that regulate cell growth<sup>1</sup>. MAP kinase activation may result from stimulation of either tyrosine-kinase (RTK) receptors, which possess intrinsic tyrosine kinase activity, or G-protein-coupled receptors (GPCR)<sup>2–4</sup>. RTK-mediated mitogenic signalling involves a series of SH2- and SH3-dependent protein-protein interactions between tyrosine-phosphorylated receptor, Shc, Grb2 and Sos, resulting in *Ras*-dependent MAP kinase activation<sup>5–7</sup>. The  $\beta\gamma$  subunits of heterotrimeric G proteins (G $\beta\gamma$ ) also mediate *Ras*-dependent MAP kinase activation<sup>8–10</sup> by an as-yet unknown mechanism. Here we demonstrate that activation of MAP kinase by G $\beta\gamma$ -coupled receptors is preceded by the G $\beta\gamma$ -mediated tyrosine phosphorylation of Shc, leading to an increased functional association between

Shc, Grb2 and Sos. Moreover, disruption of the Shc-Grb2-Sos complex blocks G $\beta\gamma$ -mediated MAP kinase activation, indicating that G $\beta\gamma$  does not mediate MAP kinase activation by a direct interaction with Sos. These results indicate that G $\beta\gamma$ -mediated MAP kinase activation is initiated by a tyrosine phosphorylation event and proceeds by a pathway common to both GPCRs and RTKs.

Stimulation of the epidermal growth factor (EGF) RTK results in increased tyrosine phosphorylation of both the receptor itself and Shc, and is concomitant with increased association of the receptor with Shc (Fig. 1). The Grb2 SH2-domain binds to tyrosine-phosphorylated Shc, resulting in the formation of a Shc-Grb2-Sos complex at the membrane, where Sos catalyses *Ras* guanine-nucleotide exchange (GNE)<sup>7,11,12</sup>. We measured tyrosine phosphorylation of Shc after stimulation of the G $\beta\gamma$ -coupled lysophosphatidic acid (LPA) or  $\alpha_{2A}$  adrenergic ( $\alpha_{2A}$ AR) GPCRs, or transient expression of G $\beta\gamma$  (Fig. 2). Receptor stimulation provoked a transient 3–4-fold increase in Shc phosphorylation by means of either endogenous LPA receptor or transiently expressed  $\alpha_{2A}$ AR (Fig. 2a, b).  $\alpha_{2A}$ AR-mediated Shc phosphorylation was completely blocked by pertussis toxin (PTX) treatment (Fig. 2b). Shc phosphorylation mediated by LPA or  $\alpha_{2A}$ AR, but not EGF receptor (Fig. 1b), was abolished by coexpression of a G $\beta\gamma$  binding peptide derived from  $\beta$ ARK1 ( $\beta$ 1ct)<sup>13</sup> (Fig. 2b), demonstrating that G $\beta\gamma$ -coupled receptors mediate this effect entirely through G $\beta\gamma$ . Expression of  $\beta$ 1ct has previously been shown to inhibit G $\beta\gamma$ -mediated MAP kinase activation<sup>9</sup>. Overexpression of G $\beta\gamma$  resulted in a sustained twofold increase in Shc phosphotyrosine, confirming that G $\beta\gamma$  alone is sufficient to mediate Shc phosphorylation.

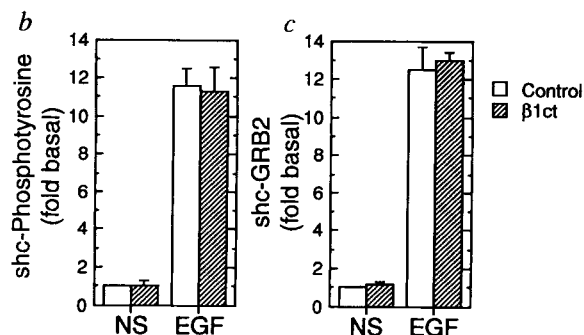
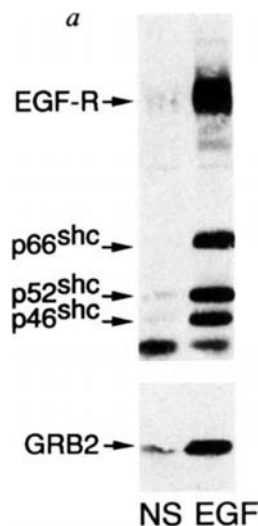
Concurrent with Shc phosphorylation, we detected an increase in Shc-Grb2 association (Figs 1a, c and 2a, c). Coexpression of  $\beta$ 1ct inhibited Shc-Grb2 complex formation mediated by the LPA and  $\alpha_{2A}$ AR receptors (Fig. 2c), but not by the EGF receptor (Fig. 1c), consistent with the effect of  $\beta$ 1ct on Shc phosphorylation.  $\alpha_{2A}$ AR-mediated phosphorylation of Shc and its subsequent association with Grb2 were rapid and transient, preceding the maximum level of MAP kinase phosphorylation (Fig. 2d).

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FIG. 1 EGF-mediated tyrosine phosphorylation of Shc, and formation of Shc-Grb2 complex. **a**, EGF-mediated increases in Shc phosphorylation as determined by anti-pTyr immunoblotting. The blot was reprobbed with anti-Grb2 polyclonal antibody to assay Shc-Grb2 complex formation. **b**, Quantification of EGF-stimulated Shc phosphorylation in the presence and absence of  $\beta 1$ ct. **c**, Quantification of EGF-stimulated Shc-Grb2 complex formation in the presence and absence of  $\beta 1$ ct.

**METHODS.** **a**, **b**, Shc was immunoprecipitated from COS-7 cells stimulated for 2 min by EGF, and Shc pTyr-phosphorylation was measured. Cell lysates were prepared in ice-cold RIPA buffer (150 mM NaCl, 50 mM Tris, pH 7.5, 0.25% deoxycholate, 0.1% Nonidet P-40, 1 mM  $\text{NaVO}_4$ , 1 mM PMSF, 1 mM NaF,  $10 \mu\text{g ml}^{-1}$  aprotinin,  $10 \mu\text{g ml}^{-1}$  leupeptin), sonicated briefly and clarified by centrifugation. Endogenous Shc was immunoprecipitated by using anti-Shc polyclonal antibody (Upstate Biotechnology, NY). Shc phosphorylation was measured by immunoblotting with mouse monoclonal anti-phosphotyrosine antibody (Upstate Biotechnology Inc.) and sheep anti-mouse  $^{125}\text{I}$ -labelled Fab (Amersham) as previously described<sup>25</sup> (upper panel), and quantified with a Molecular Dynamics phosphorimager. **a**, **c**, Immunoblots were reprobbed with mouse monoclonal anti-Grb2 (Transduction Laboratories), detected by enzyme-linked chemiluminescence (ECL,



Amersham) and quantified by laser densitometry (lower panel). Final concentrations of epidermal growth factor (EGF; Sigma),  $10 \text{ ng ml}^{-1}$ ; NS, non-stimulated. The data shown represent the means  $\pm$  s.d. from 3 similar independent experiments.

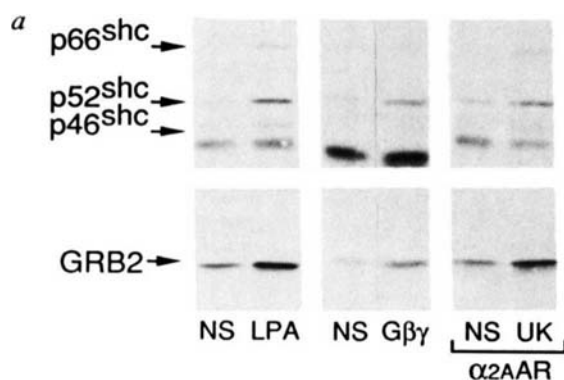
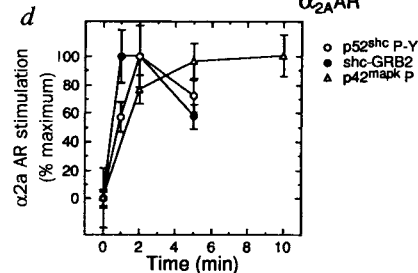
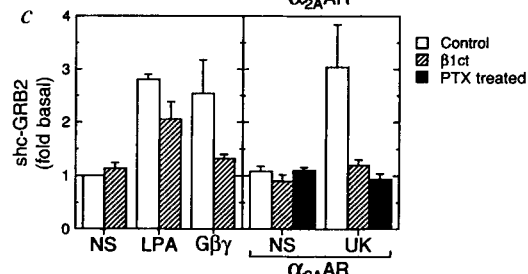
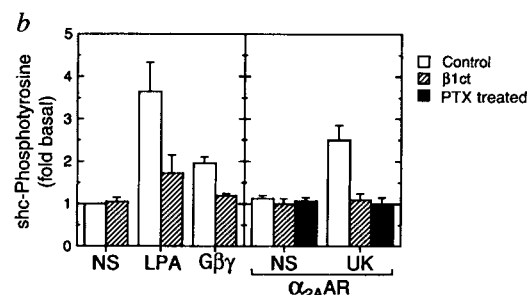
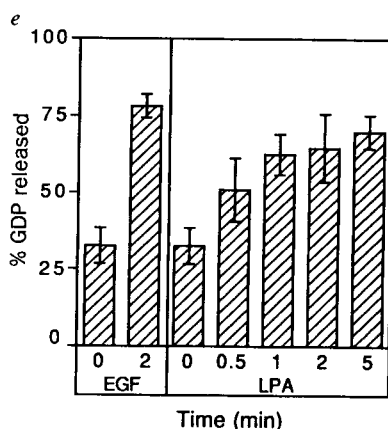


FIG. 2  $\text{G}\beta\gamma$ -mediated Shc phosphorylation and Shc-Grb2-Sos complex formation. **a**,  $\text{G}\beta\gamma$ -mediated increase in Shc phosphorylation as determined by anti-pTyr immunoblotting. COS-7 cells transfected with  $\alpha_2\text{AAR}$ ,  $\text{G}\beta 1$  and  $\text{G}\gamma 2$  cDNAs ( $\text{G}\beta\gamma$ ), or vector alone (LPA) were assayed for Shc phosphorylation content. The blot was reprobbed with anti-Grb2 polyclonal antibody to assay Shc-Grb2 complex formation. **b**, Quantification of  $\text{G}\beta\gamma$ -stimulated Shc phosphorylation in the presence and absence of  $\beta 1$ ct and PTX. **c**, Quantification of  $\text{G}\beta\gamma$ -stimulated Shc-Grb2 complex formation in the presence and absence of  $\beta 1$ ct and PTX. **d**, Time course of Shc phosphorylation, Shc-Grb2 complex formation and MAP kinase phosphorylation. **e**, Guanine-nucleotide-exchange factor activity associated with Shc immunoprecipitates from cells overexpressing mSos1.

**METHODS.** **a**–**c**, Shc was immunoprecipitated from quiescent basal and agonist-stimulated (2 min) COS-7 cells (LPA,  $\alpha_2\text{AAR}$ ) or control and transfected<sup>27</sup> cells ( $\text{G}\beta\gamma$ ). Assays were performed and quantified as described in Fig. 1. **d**,  $\alpha_2\text{AAR}$  receptor was stimulated for the indicated times. MAP kinase phosphorylation was assayed as previously described<sup>9</sup>. **e**, Shc was immunoprecipitated from cell lysates prepared in 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 2.5 mM  $\text{MgCl}_2$ , 1 mM EGTA, 1 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitors. Immunoprecipitates were washed with Tris buffer (20 mM



Tris-Cl, pH 7.5, 50 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT) and resuspended in assay buffer (Tris buffer containing  $1 \text{ mg ml}^{-1}$  BSA, 0.6% n-octylglucopyranoside, 0.1 mM GTP- $\gamma\text{S}$ , 0.8 mM AMP-PNP, 10 mM NaF, 1 mM  $\text{Na}_3\text{VO}_4$ , and protease inhibitors). Prenylated K-Ras ( $0.4 \mu\text{M}$ ) was loaded with [ $^3\text{H}$ ]GDP ( $2 \mu\text{M}$ ) as described<sup>26</sup> and added to the bead slurry (150 nM final concentration). Samples were incubated at room temperature for 30 min. The quantity of [ $^3\text{H}$ ]GDP remaining bound to K-Ras was determined as described<sup>26</sup>. Percentage GDP (ordinate) is shown as  $100 - \% \text{GDP bound to K-Ras}$ . Final concentrations of agonists: EGF (Sigma),  $10 \text{ ng ml}^{-1}$ ; lysophosphatidic acid (LPA; Sigma),  $10 \mu\text{M}$ ; UK14304 (UK; Pfizer),  $10 \mu\text{M}$ .

To determine whether Shc phosphorylation resulted in recruitment of Sos, we measured the GNE activity associated with Shc immunoprecipitates. The increase in Shc-associated GNE activity in response to either EGF or LPA was maximal within 1 minute of stimulation (Fig. 2e), thus preceding peak phosphorylation of MAP kinase. These data are consistent with the role of the Shc-Grb2-Sos complex as an upstream effector of MAP kinase activation in both the RTK and G $\beta\gamma$  signalling pathways.

To examine the role of Sos in G $\beta\gamma$ -mediated MAP kinase activation, we determined whether disruption of the Shc-Grb2-

Sos complex would inhibit the G $\beta\gamma$  signal. The proline-rich carboxy-terminal fragment of mSos1 (Sos-Pro), when expressed in COS-7 cells, co-immunoprecipitated with endogenous Grb2 (Fig. 3a), indicating that this peptide contains the determinants required for Grb2 binding. A second mutant of mSos1 ( $\Delta$ mSos1), which has a deletion of the GNE domain<sup>14</sup>, also co-immunoprecipitated with Grb2. Increasing expression of Sos-Pro progressively blocked the association of mSos1 with endogenous Shc, thereby inhibiting the formation of functional Shc-Grb2-Sos complexes (Fig. 3b).

Transiently expressed Sos-Pro and  $\Delta$ mSos1 blocked EGF-

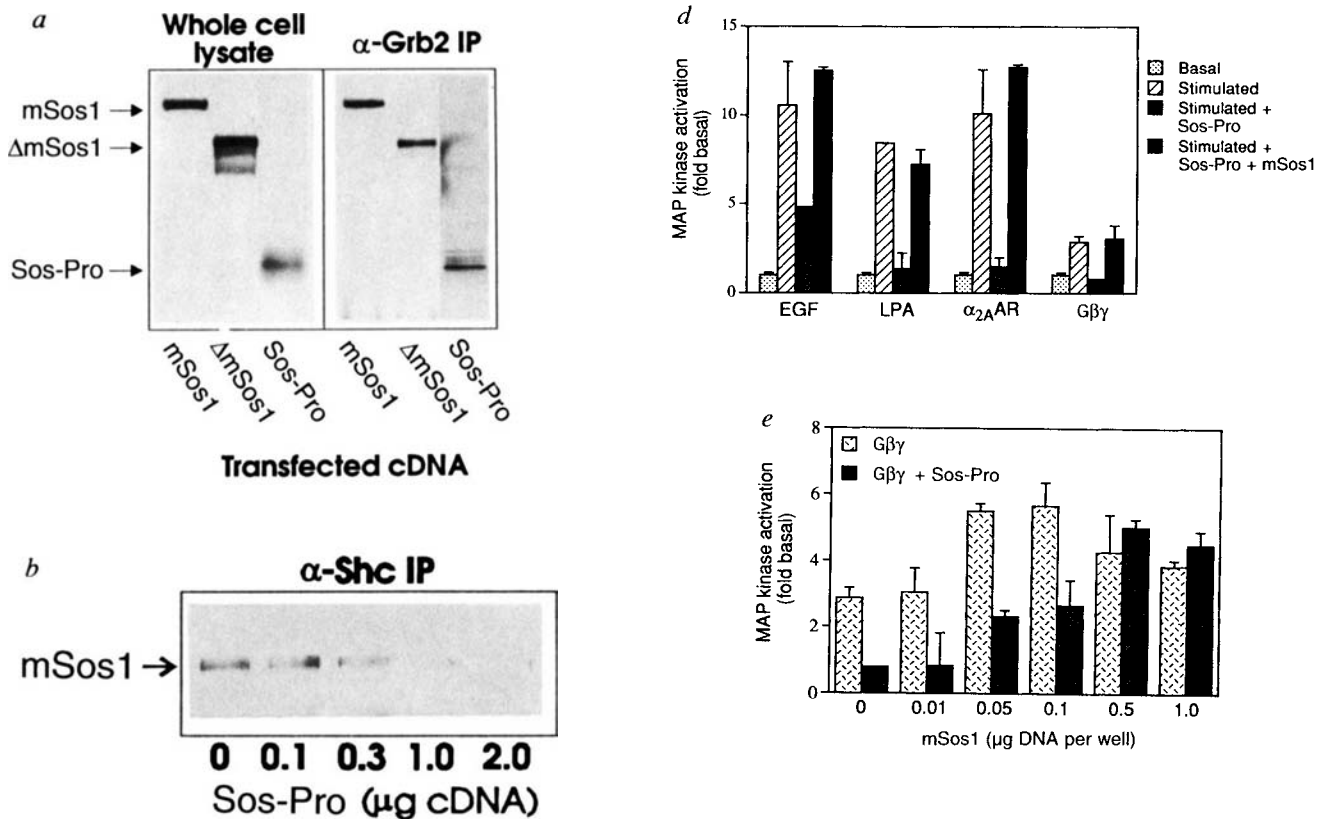


FIG. 3 Inhibition of MAP kinase activity by Sos-Pro and  $\Delta$ mSos1. **a**, Binding of mSos1,  $\Delta$ mSos1, and Sos-Pro to endogenous Grb2. Left, Immunodetection of expressed proteins; right, co-immunoprecipitation of mSos1,  $\Delta$ mSos1 and Sos-Pro with endogenous Grb2. **b**, Competitive inhibition of Shc-Grb2-mSos1 complex formation by Sos-Pro. **c**, Inhibition of MAP kinase activation by  $\Delta$ mSos1 and Sos-Pro. **d**, Wild-type mSos1 overcomes the  $\Delta$ mSos1- and Sos-Pro-mediated inhibition of MAP kinase. **e**, Sos-Pro-mediated inhibition of G $\beta\gamma$ -stimulated MAP kinase activity is restored by wild-type mSos1 in a dose-dependent manner.

**METHODS.** **a**, Left, COS-7 cells transfected with the indicated cDNA were immunoblotted with anti-mSos1 polyclonal antibody; right, endogenous Grb2 was immunoprecipitated from Cos-7 cells transfected with the indicated plasmid DNA using anti-Grb2 monoclonal antibody. mSos1 was detected with anti-mSos1 polyclonal antibody. **b**, COS-7 cells were co-transfected with 0.3  $\mu$ g mSos1 cDNA and the indicated amount of Sos-Pro cDNA. Shc was immunoprecipitated using anti-Shc polyclonal antibody and mSos1 was detected as described above. **c**, COS-7 cells were transfected with p44-HA<sup>ERK1</sup> plus  $\alpha_2AAR$ , G $\beta\gamma$  and G $\gamma_2$  cDNAs (G $\beta\gamma$ ), pRS-T24Ras (T24Ras), or vector alone (EGF and LPA). Where indicated,  $\Delta$ mSos1 and Sos-Pro were co-transfected with a 5-fold excess of DNA. MAP kinase activity was determined in basal and agonist-stimulated (5 min) cells (EGF, LPA,  $\alpha_2AAR$ ) or control and transfected cells (G $\beta\gamma$  and T24Ras). p44-HA<sup>ERK1</sup> activity was measured as previously described<sup>25</sup>. Inset, MAP kinase phosphorylation assay was

performed as previously described<sup>9</sup>, except that HA-ERK1 was immunoprecipitated with 12CA5 antibody and immunoblotted with rabbit polyclonal anti-ERK1 antibody. Phosphorylated HA-ERK1 is labelled HA-ERK1\*. **d**, COS-7 cells were transfected with p44-HA<sup>ERK1</sup> plus either  $\alpha_2AAR$ , G $\beta\gamma$  or vector alone (EGF and LPA). Cells were co-transfected with either Sos-Pro alone or Sos-Pro and SR $\alpha$ Sos, stimulated for 5 min and p44-HA<sup>ERK1</sup> activity was determined. **e**, COS-7 cells were co-transfected with p44-HA<sup>ERK1</sup>, G $\beta\gamma$ , either 1  $\mu$ g Sos-Pro or vector DNA, and increasing amounts of pRS-mSos1 as indicated. p44-HA<sup>ERK1</sup> activity was assayed as described. Values shown represent the means  $\pm$  s.d. of duplicate samples from a representative experiment.

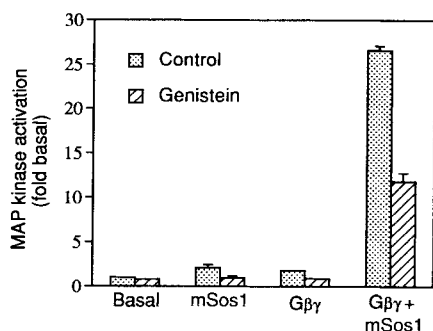


FIG. 4 mSos1 potentiates the Gβγ-mediated stimulation of MAP kinase activity in CHO cells. CHO cells were co-transfected with Gβγ or SRαSos, or an equal amount of each. All cells were co-transfected with p44-HA<sup>ERK1</sup>. Cells were treated with 100 μM Genistein (Sigma) for 2 h before cell harvesting, as indicated. Quiescent cells were collected and MAP kinase activity was assayed as described. Values shown represent the means ± s.d. of duplicate samples from a representative experiment. Similar results were obtained in 3 or more independent experiments.

mediated MAP kinase activation (Fig. 3c). Activation of MAP kinase by Gβγ, or by the LPA and α<sub>2A</sub>AR receptors, was abolished by both dominant negative mutants of mSos1, whereas MAP kinase activation by constitutively active Ras (T24Ras) (ref. 15) was unaffected. These data indicate that Sos-Pro and ΔmSos1 block MAP kinase activation mediated directly by Gβγ subunits, by GPCRs that signal through Gβγ, and by RTKs, without affecting signalling downstream of Ras.

In cells inhibited by Sos-Pro or ΔmSos1, MAP kinase activation by either the EGF receptor or the α<sub>2A</sub>AR and LPA receptors was fully restored by coexpressed mSos1 (Fig. 3d). Expression of mSos1 alone caused a small (less than twofold) increase in MAP kinase activity (results not shown). In cells expressing Gβγ and Sos-Pro, mSos1 overcame the Sos-Pro-mediated inhibition of MAP kinase in a dose-dependent manner (Fig. 3e), indicating that Sos-Pro competes with wild-type Sos for Shc-Grb2-Sos complex formation.

Several proteins involved in Ras activation contain pleckstrin-homology (PH) domains, including the GNE factors Sos and GRF, and the GTPase-activating protein rasGAP<sup>16</sup>. The PH domains of several proteins bind Gβγ *in vitro*<sup>17,18</sup>, and the C-terminal PH domain of βARK1 mediates the Gβγ-dependent translocation of βARK1 to the membrane<sup>19</sup>. Because membrane association of Sos is sufficient for its activation<sup>20</sup>, Gβγ subunits might activate Ras by a Sos PH-domain-directed translocation event. If so, the Gβγ pathway would be expected to circumvent Shc and Grb2. However, because disruption of the Shc-Grb2-Sos interaction by Sos-Pro blocks MAP kinase activation, it appears that an association between Grb2-Sos and Shc is needed

to transmit the Gβγ signal to MAP kinase. This indicates that a PH-domain-directed interaction between Gβγ and Sos is not sufficient for MAP kinase activation.

In CHO cells, overexpressed Gβγ and mSos1 acted synergistically to activate MAP kinase (Fig. 4). Transient expression of either Gβγ or mSos1 alone elicited a modest two- to threefold increase in MAP kinase activity. When mSos1 was coexpressed with Gβγ, MAP kinase activity increased by >25-fold. In each case, MAP kinase activation was attenuated by exposure of the cells to the protein-tyrosine-kinase (PTK) inhibitor genistein (Fig. 4). In contrast, T24Ras-mediated MAP kinase activation was insensitive to genistein (results not shown). These data suggest that Gβγ-mediated MAP kinase activation requires Sos and is dependent upon a tyrosine-phosphorylation event. Genistein and herbimycin A, a Src family-specific PTK inhibitor, have been shown to inhibit MAP kinase activation by Gβγ (ref. 21) or by G<sub>i</sub>-coupled receptors<sup>22</sup>. The G<sub>i</sub>-coupled thrombin receptor has been shown to mediate the autophosphorylation and activation of pp60c-Src in a rapid and transient manner<sup>23</sup>, suggesting that pp60c-Src, or a Src-family PTK, may phosphorylate Shc in response to a Gβγ-mediated signal. Lev *et al.*<sup>24</sup> describe a novel Ca<sup>2+</sup>-sensitive PTK, PYK2, which is activated by a variety of stimuli, including the GPCR for bradykinin.

We show that Gβγ-mediated tyrosine phosphorylation of Shc is among the earliest detectable events in this pathway, followed by a signalling cascade that proceeds through Grb2, Sos and Ras to MAP kinase activation. Strikingly, this pathway involves all the same intermediates that function in the RTK mitogenic signalling pathway, including at least one PTK. □

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# Activation of the c-Abl tyrosine kinase in the stress response to DNA-damaging agents

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**THE product of the *c-abl* gene is a non-receptor tyrosine kinase that is localized to the nucleus and cytoplasm. The precise function of c-Abl is unknown. Here we show that ionizing radiation activates c-Abl. Similar results were obtained with the alkylating agents *cis*-platinum and mitomycin C. We also demonstrate that cells deficient in c-Abl fail to activate Jun kinase (JNK/SAP kinase) after ionizing radiation or alkylating agent exposure and that reconstitution of c-Abl in these cells restores that response. In contrast, the stress response to tumour-necrosis factor is stimulated by a c-Abl-independent mechanism. These findings indicate that *c-abl* is involved in the stress response to DNA-damaging agents.**

Human U-937 cells were exposed to 2 Gy of ionizing radiation and collected 1 hour later. Nuclear lysates were subjected to immunoprecipitation with anti-Abl antibody and the immunoprecipitates were assayed for phosphorylation of a GST-Crk fusion protein. The c-Crk protein contains an amino-terminal SH2 domain followed by two SH3 domains. c-Abl binds to the N-terminal SH3 domain of Crk and phosphorylates Tyr 221 (refs 1, 2). There was a low level of GST-Crk(120-225) phosphorylation with anti-Abl immunoprecipitates from control cells, whereas exposure to ionizing radiation was associated with stimulation (four- to fivefold) of Crk kinase activity (Fig. 1a). In contrast, there was no detectable ionizing-radiation-induced phosphorylation of a GST-Crk(120-212) fusion protein that lacks Tyr 221 (Fig. 1a). The finding that ionizing radiation has no detectable effect on c-Abl levels supported an increase in c-Abl activity (Fig. 1a). NIH3T3 fibroblasts were used in similar experiments to determine whether activation of c-Abl is detectable in different cell types. The results show that treatment with ionizing radiation is also associated with stimulation of c-Abl activity in these cells (Fig. 1a). To assess this activation of c-Abl further, we used a peptide (EAIYAAPFAKKK) recently identified as a specific substrate for c-Abl activity<sup>3</sup>. Anti-Abl immunoprecipitates from irradiated U-937 cells exhibited maximal (nearly fivefold) phosphorylation of the peptide substrate 1 hour later (Fig. 1b). In contrast, immunoprecipitates prepared with preimmune rabbit serum did not have phosphorylation of the peptide induced by ionizing radiation (Fig. 1b). Because ionizing radiation induces single and double DNA strand breaks, we investigated whether treatment with other agents that damage DNA is also associated with c-Abl activation. *Cis*-platinum (CDDP) forms DNA-intrastrand crosslinks<sup>4</sup>, whereas mitomycin C (MMC) forms monofunctional and bifunctional DNA lesions<sup>5</sup>. Treatment of NIH3T3 cells with these alkylating agents was associated with an increase in c-Abl activity that was similar to that obtained after exposure to ionizing radiation (Fig. 1c).

These findings suggest that c-Abl is activated by diverse DNA-damaging agents.

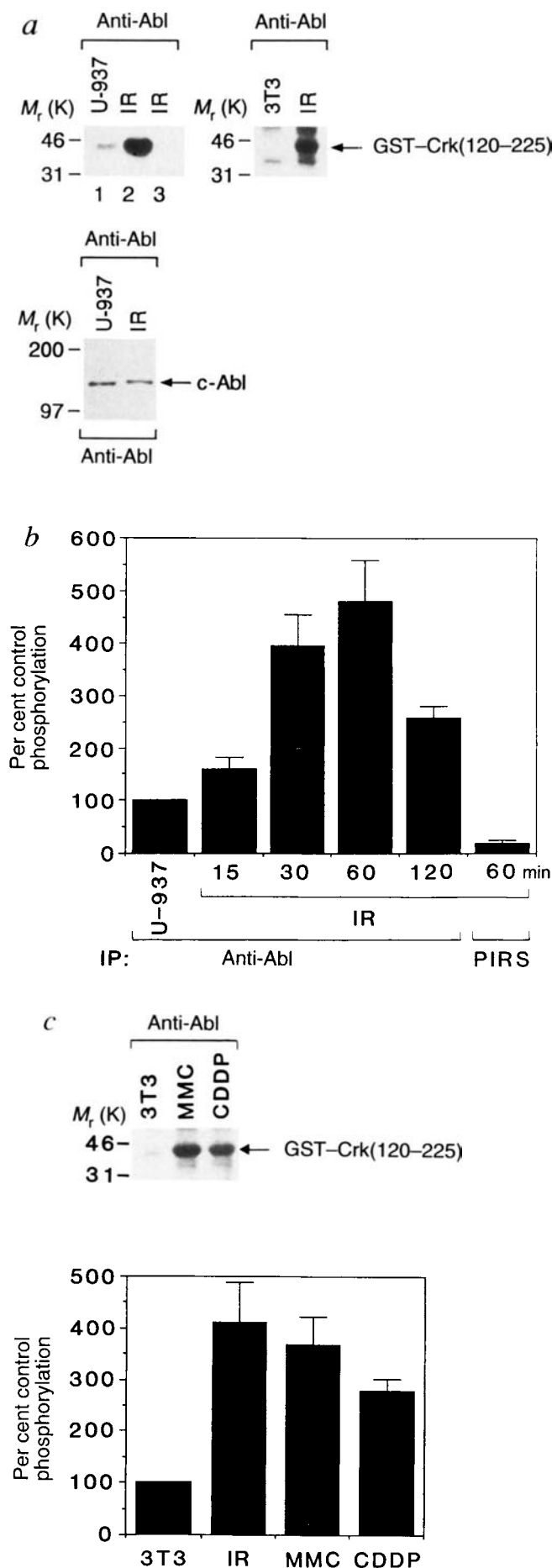
Two serines (Ser 63 and Ser 73) in the transactivation domain of c-Jun have been identified as substrates for the stress-activated protein (SAP) kinases<sup>6, 8</sup>. The SAP kinases are activated in response to treatment with tumour-necrosis factor (TNF), anisomycin and ultraviolet radiation<sup>7, 8</sup>. The finding that Ha-Ras is involved in the stimulation of SAP kinase by ultraviolet light has suggested that this signalling cascade is initiated by damage to cellular components other than DNA<sup>8, 9</sup>. Currently, little is known about the involvement of SAP kinase in the cellular response to DNA-damaging agents. To address this issue, we analysed anti-SAP kinase immunoprecipitates for phosphorylation of the transactivation domain of c-Jun. With GST-Jun(2-100) as a substrate, there was a low level of Jun phosphorylation with anti-SAP kinase immunoprecipitates from NIH3T3 cells, and this activity was increased by exposure to ionizing radiation (Fig. 2a). Similar findings were obtained with immunoprecipitates from CDDP- or MMC-treated cells (Fig. 2a). As these findings supported activation of SAP kinase by DNA-damaging agents, we investigated whether c-Abl is involved in a cascade that includes SAP kinase. Mouse fibroblasts deficient in c-Abl (Abl<sup>-/-</sup>, derived from mice with targeted *c-abl* disruption)<sup>10</sup> were assayed for SAP kinase activity after irradiation or drug treatment. In the Abl<sup>-/-</sup> cells, there was no detectable induction of SAP kinase activity after exposure to ionizing radiation, CDDP or MMC (Fig. 2b), whereas immunoblot analysis with anti-SAP kinase demonstrated expression of this protein before and after treatment of these cells (results not shown). These findings suggested that c-Abl is necessary for activation of SAP kinase in cells treated with DNA-damaging agents.

To demonstrate more definitively a role for c-Abl in a cascade that contributes to activation of SAP kinase, we stably expressed c-Abl in the Abl<sup>-/-</sup> cells (designated Abl<sup>+</sup>; R.R., unpublished data). The level of c-Abl expression in the Abl<sup>+</sup> cells was readily detectable, but somewhat lower than that in NIH3T3 cells (Fig. 3a). Ionizing irradiation of the Abl<sup>+</sup> cells was associated with stimulation of c-Abl activity (Fig. 3b). More importantly, exposure of the Abl<sup>+</sup> cells to ionizing radiation was associated with increases in SAP kinase activity (Fig. 3c). MMC treatment of the Abl<sup>+</sup> cells also resulted in activation of both c-Abl (results not shown) and SAP kinase activities (Fig. 3c). Taken together, these results provide definitive evidence that c-Abl mediates signals in the SAP kinase stress-response pathway.

The finding that DNA-damaging agents activate c-Abl suggested that this event may be a generalized response to cellular stress. Previous work has shown that the stress pathway involving SAP kinase is activated by TNF (ref. 7). However, TNF had little if any effect on c-Abl activity in NIH3T3 cells (Fig. 4a). In contrast, TNF stimulated SAP kinase activity in these cells (results not shown). Moreover, TNF induced SAP kinase activity in the Abl<sup>-/-</sup> cells (Fig. 4b). These findings suggest that c-Abl is selectively activated by agents (ionizing radiation, CDDP, MMC) that act primarily by damaging DNA, but not in the response to TNF-induced cellular stress.

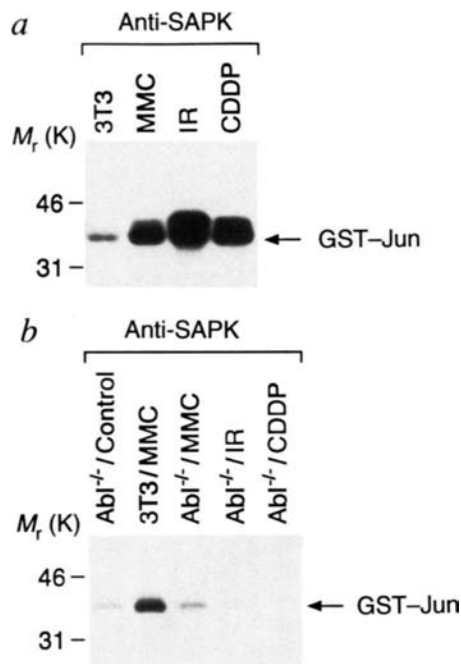
As well as sharing structural features with members of the Src family, c-Abl contains actin-binding and DNA-binding domains. The finding that c-Abl associates with the retinoblastoma protein indicates a potential role for c-Abl in regulating the cell cycle<sup>11</sup>. Other studies have shown that overexpression of c-Abl induces an arrest in G1 phase<sup>12, 13</sup>. Phosphorylation of c-Abl at multiple sites by p34<sup>cdc2</sup> during mitosis has also supported a role in G2 phase<sup>14</sup>. The phosphorylation of c-Abl in mitotic cells inhibits DNA binding<sup>15</sup>. Whereas those findings indicated that c-Abl could contribute to regulation of the cell cycle, our results show that c-Abl is activated by DNA-damaging agents. DNA damage could dissociate c-Abl from a complex with other proteins and thereby contribute to interactions with potential substrates. In this context, binding of c-Abl to the first Crk SH3 domain targets phosphorylation of c-Crk on Tyr 221



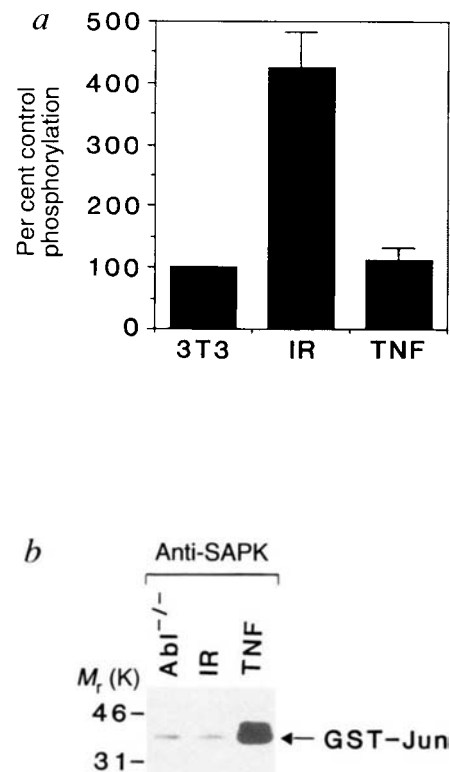


**FIG. 1** Activation of c-Abl by diverse DNA damaging agents. **a**, U-937 or NIH3T3 cells were treated with 2 Gy ionizing radiation (IR) and collected 1 h later. Nuclei were isolated and the nuclear proteins subjected to immunoprecipitation with anti-Abl (K-12, Santa Cruz Biotechnology). *In vitro* immune complex kinase assays were performed with a GST-Crk(120–225) fusion protein as substrate (U-937, left panel, lanes 1 and 2; NIH3T3, right panel). GST-Crk(120–212) fusion protein (which lacks the critical Tyr 221) was used as a negative control (lane 3). The anti-Abl immunoprecipitates were also analysed by immunoblotting with anti-Abl (bottom panel). **b**, U-937 cells treated with 2 Gy of ionizing radiation (IR) and collected after the indicated times. Nuclear proteins were then subjected to immunoprecipitation with anti-Abl antibody. Immunoprecipitations were also performed with preimmune rabbit serum (PIRS) from cells exposed to 2 Gy IR and harvested 1 h later. *In vitro* immune complex kinase assays were performed with the c-Abl substrate EAIYAAPFAKKK. The data (per cent control phosphorylation) represent the mean  $\pm$  s.e.m. of three separate experiments. **c**, NIH3T3 cells were treated with 10  $\mu$ M CDDP for 30 min, 10  $\mu$ M MMC for 1 h or 2 Gy IR (harvested 1 h later). Nuclear proteins were subjected to immunoprecipitation with anti-Abl. Kinase assays were performed with GST-Crk(120–225) fusion protein (upper panel) or EAIYAAPFAKKK peptide (lower panel) as substrates.

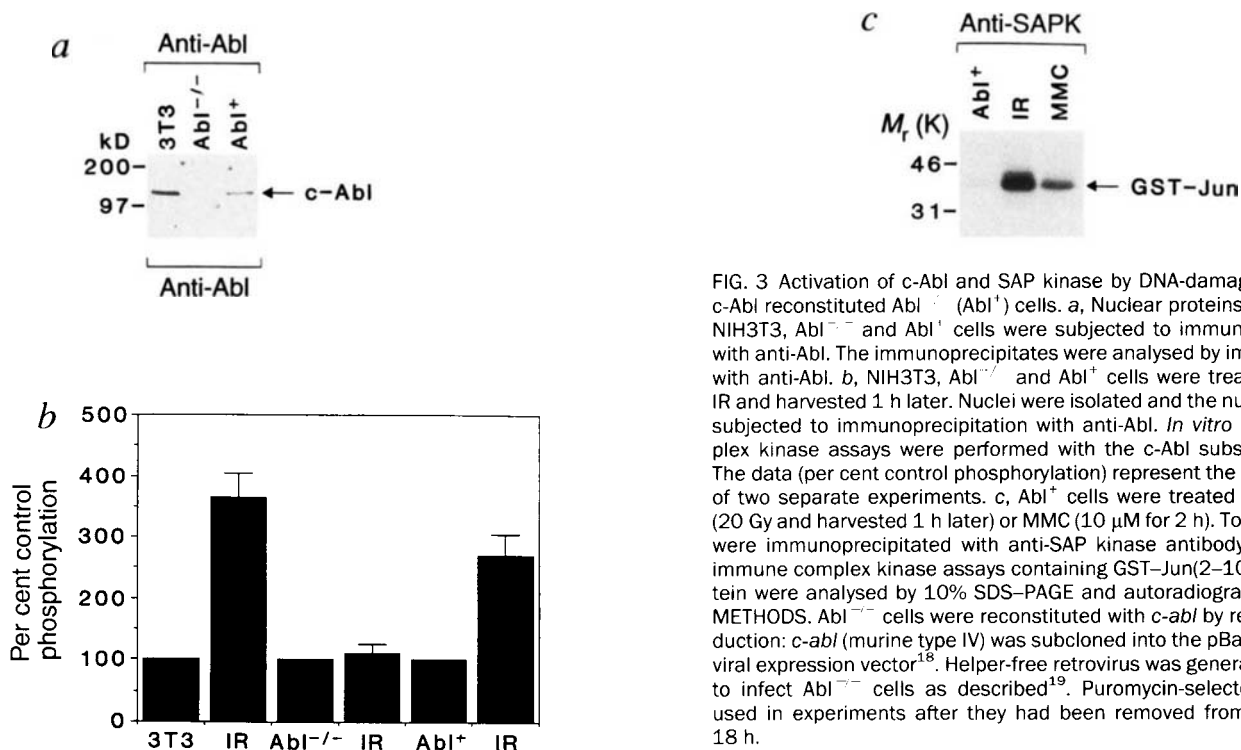
**METHODS.** U-937 and NIH3T3 cells were treated with 2 Gy IR at room temperature with a Gammacell 1000 (Atomic Energy of Canada, Ottawa) under aerobic conditions with a  $^{137}\text{Cs}$  source emitting at a fixed dose rate of 0.76 Gy min $^{-1}$  as determined by dosimetry. The cells were then incubated for the indicated times. They were also treated with 10  $\mu$ M mitomycin C (MMC; Sigma for 1 h or 10  $\mu$ M cis-platinum (CDDP) for 30 min. The cells were swelled in 2 ml of ice-cold hypotonic lysis buffer (1 mM EGTA, 1 mM EDTA, 10 mM  $\beta$ -glycerophosphate, 2 mM MgCl $_2$ , 10 mM KCl, 1 mM sodium orthovanadate, 1 mM phenylmethylsulphonyl fluoride, 1 mM DTT, 10  $\mu$ g ml $^{-1}$  each of pepstatin, leupeptin and aprotinin) for 30 min and then subjected to Dounce homogenization (15–25 strokes, tight pestle A). The resulting lysate was loaded onto 1.5 ml of buffer A (1 M sucrose in hypotonic lysis buffer containing the protease and phosphatase inhibitors) and centrifuged at 1,600g for 15 min to pellet nuclei. The pellet was washed and solubilized in buffer A containing 1% (v/v) NP-40. Anti-c-Abl immunoprecipitations were performed by adding the K12 anti-Abl antibody and protein A-Sepharose for 2 h at 4  $^{\circ}$ C. Immune complex kinase assays were performed by incubating the resulting protein complexes in kinase buffer (50 mM Tris, pH 7.5, 10 mM MnCl $_2$ , 1 mM dithiothreitol), with either 5  $\mu$ g GST-Crk(120–225) or GST-Crk(120–212), 2–5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP (New England Nuclear) for 30 min at 28  $^{\circ}$ C and analysed by 10% SDS-PAGE and autoradiography. In the peptide phosphorylation assays, immune complexes were incubated in kinase buffer with 20  $\mu$ M peptide [EAIYAAPFAKKK], 10  $\mu$ M ATP and 2–5  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP for 4 min at 25  $^{\circ}$ C. After incubation, 25  $\mu$ l aliquots were spotted onto phosphocellulose discs, followed by washing with 1% (v/v) phosphoric acid and then distilled water. The incorporated  $^{32}$ P was determined by scintillation counting.



**FIG. 2** Activation of SAP kinase activity by DNA damaging agents. **a**, NIH3T3 cells were treated with 20 Gy IR (harvested 1 h later), 10  $\mu$ M CDDP for 2 h or 10  $\mu$ M MMC for 2 h. Total lysates were immunoprecipitated with anti-SAP kinase antibody, and *in vitro* immune complex kinase reactions containing GST-Jun(2–100) fusion protein were analysed by 10% SDS-PAGE and autoradiography. **b**, Abl<sup>-/-</sup> cells were treated with 20 Gy IR (harvested 1 h later), 10  $\mu$ M CDDP for 2 h or 10  $\mu$ M MMC for 2 h. NIH3T3 cells were also treated with 10  $\mu$ M MMC for 2 h as a positive control. Total cell lysates were immunoprecipitated with anti-SAP kinase, and *in vitro* immune complex kinase assays were performed with GST-Jun(2–100) as substrate. **METHODS**. GST-Jun(2–100) fusion protein was prepared as described<sup>17</sup>. The Abl<sup>-/-</sup> fibroblast cell line was obtained from D. Baltimore's laboratory (Massachusetts Institute of Technology, Cambridge, MA).



**FIG. 4** Activation of SAP kinase by TNF is independent of c-Abl. **a**, NIH3T3 cells were treated with 2 Gy IR (harvested 1 h later) or 10 ng ml<sup>-1</sup> TNF for 15 min. Nuclear lysates were immunoprecipitated with anti-Abl antibody, and *in vitro* immune complex kinase assays were performed with peptide as substrate. **b**, NIH3T3 or Abl<sup>-/-</sup> cells were treated with 2 Gy IR for 30 min or 20 Gy IR. Total cell lysates were immunoprecipitated with anti-SAP kinase, and immune complex kinase assays were performed with GST-Jun(2–100) as substrate.



**FIG. 3** Activation of c-Abl and SAP kinase by DNA-damaging agents in c-Abl reconstituted Abl<sup>-/-</sup> (Abl<sup>+</sup>) cells. **a**, Nuclear proteins isolated from NIH3T3, Abl<sup>-/-</sup> and Abl<sup>+</sup> cells were subjected to immunoprecipitation with anti-Abl. The immunoprecipitates were analysed by immunoblotting with anti-Abl. **b**, NIH3T3, Abl<sup>-/-</sup> and Abl<sup>+</sup> cells were treated with 2 Gy IR and harvested 1 h later. Nuclei were isolated and the nuclear proteins subjected to immunoprecipitation with anti-Abl. *In vitro* immune complex kinase assays were performed with the c-Abl substrate peptide. The data (per cent control phosphorylation) represent the mean  $\pm$  s.e.m. of two separate experiments. **c**, Abl<sup>+</sup> cells were treated with either IR (20 Gy and harvested 1 h later) or MMC (10  $\mu$ M for 2 h). Total cell lysates were immunoprecipitated with anti-SAP kinase antibody, and *in vitro* immune complex kinase assays containing GST-Jun(2–100) fusion protein were analysed by 10% SDS-PAGE and autoradiography. **METHODS**. Abl<sup>-/-</sup> cells were reconstituted with c-abl by retroviral transduction: c-abl (murine type IV) was subcloned into the pBaBe-puro retroviral expression vector<sup>18</sup>. Helper-free retrovirus was generated and used to infect Abl<sup>-/-</sup> cells as described<sup>19</sup>. Puromycin-selected cells were used in experiments after they had been removed from the drug for 18 h.

(refs 1, 2, 16). Because DNA damage is associated with arrest of cells in G1 and G2 phases, c-Abl activation could play a role in regulating these responses to genotoxic stress. Alternatively, whereas overexpression of c-Abl arrests cells in G1 phase<sup>12,13</sup>, activation of c-Abl by DNA damage may regulate distinct stress pathways that include SAP kinase. □

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## Structure of guanine-nucleotide-exchange factor human Mss4 and identification of its Rab-interacting surface

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**GUANINE-NUCLEOTIDE-EXCHANGE factors (GEFs) promote the exchange of GDP for GTP in Ras GTPases, and thereby positively regulate their functions<sup>1,2</sup>. Members of the Sec4/Ypt1/Rab branch of the Ras superfamily are essential for vesicular transport<sup>3–6</sup>. A GEF for a subset of Rab proteins, termed mammalian suppressor of Sec4 (Mss4), has been identified<sup>7</sup>. Here we use multidimensional NMR to determine the structure of human Mss4 (hMss4), which is the first tertiary structure established for a protein with GEF activity. Mss4 contains a central  $\beta$ -sheet sandwiched between two small sheets. It also binds a Zn<sup>2+</sup> ion through Cys 23, Cys 26, Cys 94 and Cys 97. The Rab-binding surface of hMss4 has subsequently been delineated using chemical-shift perturbation experiments and site-directed mutagenesis. The active site of hMss4 involves the Zn<sup>2+</sup>-binding region and a neighbouring loop.**

hMss4 can be described as a three-layered  $\beta$  protein with a central seven-stranded antiparallel sheet flanked by a small three-stranded sheet on one face and a  $\beta$  hairpin on the other (Figs 1a, 2a). Most side chains located at both faces of the central sheet are hydrophobic. Residues of the top face, including His 68, Leu 70, Ile 89, Phe 91, Val 93, Trp 104, Tyr 114 and Ala 116, are shielded from the solvent by strands  $\beta$ E and  $\beta$ F,

particularly sidechains from Leu 43, Leu 45, Pro 46, Met 48 and Leu 65. The sidechain of His 68 is neutral and packs against Leu 43, Leu 65 and Tyr 114 (Fig. 1b). The three-stranded sheet, the  $3_{10}$  helix A, and the amino-terminal loop cap the bottom face of the central sheet, enclosing a hydrophobic core. Another noteworthy feature of hMss4 is that Pro 101 adopts the *cis* conformation, putting its side chain, like that of Pro 46, in close proximity to the aromatic ring of Phe 91. We have recently reported that hMss4 binds a zinc ion<sup>8</sup>. Our structure now reveals that the Zn<sup>2+</sup> ion is coordinated tetrahedrally by Cys 23, Cys 26, Cys 94 and Cys 97, which pairwise comprise the two CysXXCys motifs of hMss4 (Fig. 2). Close to the Zn<sup>2+</sup> site is the loop connecting  $\beta$ E and  $\beta$ F (EF loop). This loop appears to be highly dynamic, as the amide <sup>1</sup>H/<sup>15</sup>N correlation of many residues in the region could not be observed in a heteronuclear single quantum coherence (HSQC) spectrum<sup>9</sup>.

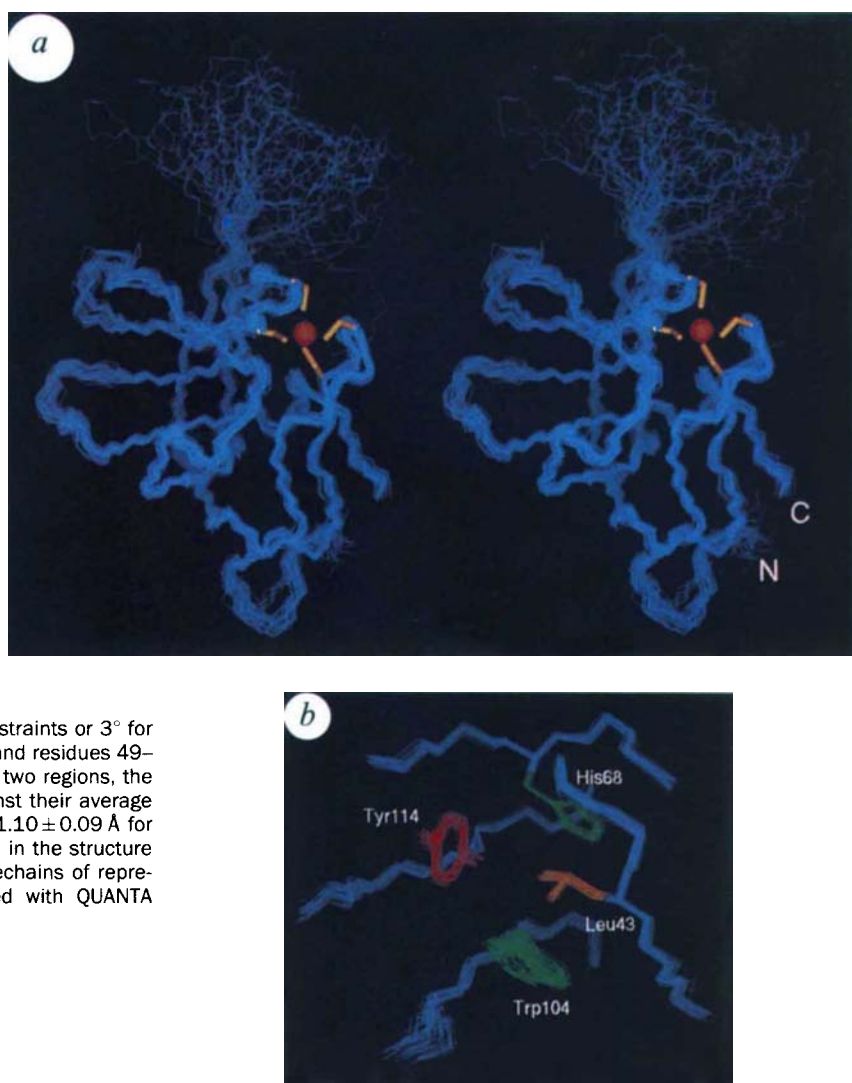
Neither Mss4 nor its yeast homologue DSS4 has significant sequence similarities with GEFs for Ras, Ran or Rho<sup>1,7,10</sup>. Even the sequence identities between Mss4 and DSS4 are very localized (Fig. 2b). Specifically, one of the Zn<sup>2+</sup>-binding loops (the IJ loop) and the GH loop (including the  $3_{10}$  helix A) of hMss4 show the highest sequence similarity to DSS4. The two loops are close and interact with each other (Fig. 3a). Because Mss4 and DSS4 can promote GDP–GTP exchange in a common set of Rabs, these conserved regions may define the active site. This speculation is indeed confirmed by our results from chemical-shift perturbation studies and structure-based mutagenesis on hMss4.

GEFs seem to exert their functions by stabilizing the nucleotide-free form of Ras proteins<sup>1,11,12</sup>. Mss4 binds to Rab3a tightly in the absence of GTP or GDP, whereas a large excess of GTP or GDP dissociates the complex<sup>13</sup>. Based on these observations, we prepared a nucleotide-free form of Sec4 (refs 8, 12) and examined its interactions with hMss4 by NMR. The nucleotide-free form of Sec4 was obtained by dialysing for at least 6 days the purified recombinant Sec4 protein against a buffer that did not contain GTP or GDP. Addition of 0.5 equivalents of Sec4 into a 1 mM solution of <sup>15</sup>N-labelled hMss4 caused the intensities of all signals in an HSQC spectrum to decrease by ~50%, whereas chemical shifts and linewidths of these signals remained the same as those of the free hMss4 (results not shown). The sharp resonances of free hMss4 disappeared after the further addition of another 0.5 equivalent of Sec4. Meanwhile, a set of signals with broader linewidth, presumably belonging to the complex, were observed. Therefore Mss4 associates with Sec4 tightly enough to yield a slowly exchanging complex on the NMR timescale<sup>14</sup>. If the on-rate (*k*<sub>on</sub>) is limited by diffusion, the dissociation constant (*K*<sub>d</sub>) of the Mss4–Sec4 complex is probably <1  $\mu$ M for their binding to be a slow exchanging process. Most of the Sec4-bound hMss4 signals have <sup>1</sup>H and <sup>15</sup>N chemical shifts nearly identical to those of the free hMss4. However, dramatic <sup>1</sup>H or <sup>15</sup>N chemical-shift perturbations of the amide groups were found for Asn 17, Gln 24, Arg 25, Val 30, Val 71, Phe 75, Asn 79, Gly 81, Phe 82, Thr 83, Phe 91, Leu 92, Val 93, Cys 94 and Ala 95. Most of these residues are located at the GH and IJ loops, indicating that hMss4 interacts with Sec4 through these two loops (Fig. 3).

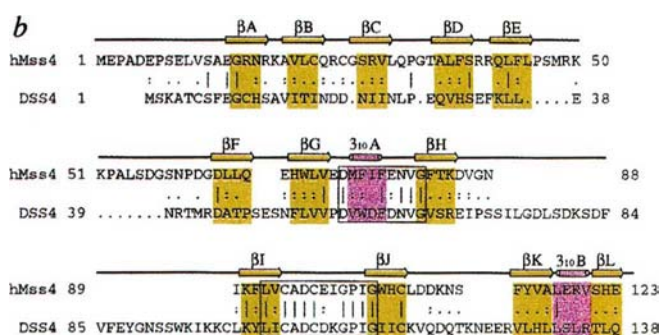
We also constructed several hMss4 mutants that target conserved residues in this region<sup>15</sup>, including N79D, A95V, D96H, E98K, and a mutant with part of the EF loop (residues 49–54) deleted. The deletion mutant was made because the EF loop is near the Zn<sup>2+</sup>-binding site and the amide <sup>1</sup>H/<sup>15</sup>N correlation of many residues in the loop cannot be observed in the HSQC spectrum. Therefore our chemical shift perturbation experiments did not address whether this loop is involved in binding to Sec4. The GEF activities of these mutants were then assayed as described previously using the Sec4 protein as substrate<sup>8</sup>. Both the N79D and D96H mutants retain only 40% GEF activity compared with that of the wild-type hMss4 (results not shown). Consistent with this observation, one of the side-chain amide

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**FIG. 1** Stereo view of the 30 overlaid NMR structures of hMss4. **a**, Backbone (N, C $\alpha$ , C) traces of the 30 final structures. The zinc ion is shown as a red sphere; its coordinating cysteine residues are displayed as yellow bonds. The cloning, over-expression, purification, and characterization of hMss4 have been reported elsewhere<sup>8</sup>. After making sequence-specific resonance assignments of hMss4 (ref. 8), we derived 1,289 distance restraints and 117 torsional angle restraints from nuclear Overhauser effects (NOEs) and coupling constants, respectively. The interpretation and calibration of NOEs and coupling constants were as previously described<sup>25</sup>. Initial structures calculated in the absence of zinc indicated that the zinc is coordinated tetrahedrally by the S $\gamma$  atoms of Cys 23, Cys 26, Cys 94 and Cys 97. In subsequent calculations, the zinc ion was incorporated by introducing covalent restraints for the Zn-S bonds with bond angles at 109.5° and bond lengths of 2.3 Å (ref. 26). A total of 30 structures of hMss4 were calculated using simulated annealing with the program X-PLOR 3.1 (ref. 27). None of the calculated structures shows violations >0.3 Å for the distance restraints or 3° for the dihedral restraints. The N-terminal eight residues and residues 49–62 adopt random-coil conformations. Excluding these two regions, the root-mean-square deviations of these structures against their average coordinates are  $0.62 \pm 0.08$  Å for the backbone and  $1.10 \pm 0.09$  Å for non-hydrogen atoms. Residues 1–8 were not included in the structure calculations and are not shown in the figures. **b**, Sidechains of representative core residues. The figures were generated with QUANTA (Molecular Simulations, Burlington, Massachusetts).



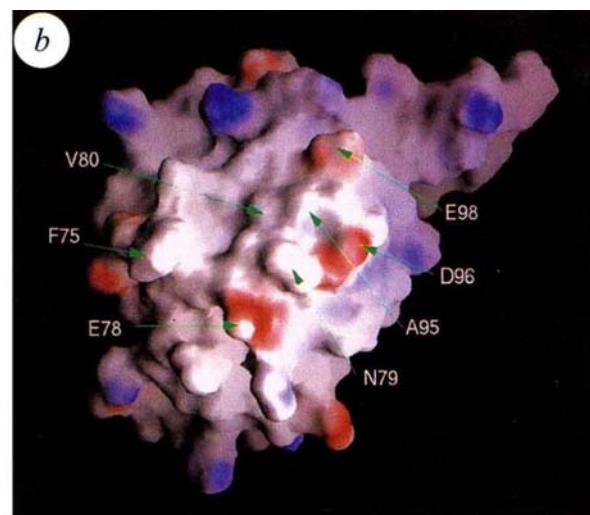
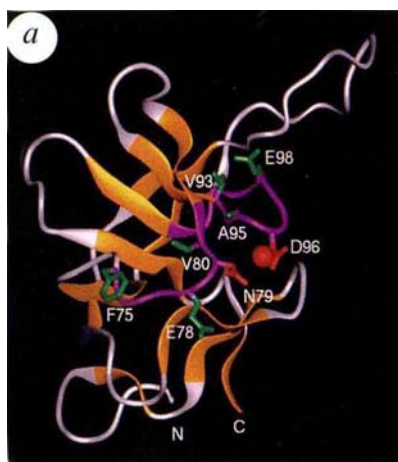
**FIG. 2** Tertiary fold and secondary structure assignment of hMss4. **a**, Ribbon diagram of hMss4 with  $\beta$ -strands as gold ribbons,  $3_{10}$  helices as purple coils, loops as white tubes, zinc as a red sphere and zinc-binding cysteines as yellow balls-and-sticks. Strands A, D, G, K, J, I and H form the central antiparallel  $\beta$ -sheet with a topology of (+1, +1, +4x, -1, -1, -1), whereas the two small sheets comprise strands (B, C, J) and strands (E, F), respectively. This drawing was generated with



RIBBONS<sup>28</sup>. **b**, Structure-based sequence alignment of hMss4 and DSS4 with the secondary-structure elements shown above. Identical residues are indicated by vertical bars. The two highly conserved regions (the GH and IJ loops) are boxed. DSS4 lacks a large portion of the EF loop although it contains a long insert in the HI loop. Because the two loops are in close proximity in the hMss4 structure, the missing residues of the EF loop in DSS4 can be partly compensated by its HI loop insert.



FIG. 3 The active site of hMss4. **a**, Ribbon diagram of hMss4 with homologous surface amino-acid side chains shown as green bonds. Asn 79 and Asp 96 are shown in red. The GH and IJ loops are shown in purple. The  $^1\text{H}$  and  $^{15}\text{N}$  chemical shifts of the amide groups of all residues shown here were perturbed on the addition of the Sec4 protein. Mutations of Asn 79 and Asp 96 also caused a dramatic decrease in the GEF activity of hMss4 toward Sec4. Generated with QUANTA. **b**, Molecular surface of hMss4. The surface is coloured according to the local electrostatic potential, with blue and red representing positive and negative charges, respectively. It shows that the conserved residues of the GH and IJ loops define a hydrophobic area lined with negative charges. Drawn with GRASP<sup>29</sup>.



protons of Asn 79 forms a hydrogen bond with the carboxyl oxygen of Asp 96, stabilizing the conformations of the GH and IJ loops. The other three mutants were found to be as active as wild-type hMss4. On this evidence we conclude that one of the  $\text{Zn}^{2+}$ -binding loops (IJ loop) and the GH loop are mainly responsible for the binding of hMss4 to Sec4 and its GEF activity. Because the deletion mutant is fully active in its ability to promote nucleotide exchange in Sec4, the EF loop does not seem to be involved in the interactions between hMss4 and Sec4.

There is a precedent for protein-protein interactions involving  $\text{Zn}^{2+}$ -binding loops. For instance, the regulatory subunit of aspartate carbamoyltransferase interacts with its catalytic subunit through two loops that coordinate a zinc ion with four cysteines<sup>16</sup>. Many proteins that bind  $\text{Zn}^{2+}$  with CysXXCys motifs contain a structure element called the rubredoxin 'knuckle', in which the sulphur of each cysteine side chain  $\text{S}_\gamma(i)$  forms a hydrogen bond with the backbone amide proton of the  $i+2$  residue<sup>17,18</sup>. In hMss4,  $\text{S}_\gamma(23)$ ,  $\text{S}_\gamma(26)$  and  $\text{S}_\gamma(94)$  form hydrogen bonds with NH(25), NH(28), and NH(96), respec-

tively (Fig. 4). The so-called 'knuckle' structure requires that the backbone is extended before and after the  $\text{Zn}^{2+}$ -binding site<sup>18</sup>. Through binding to  $\text{Zn}^{2+}$ , the conformation of the loop is thus made rigid. It may be this characteristic rigid loop structure that is required for the binding of hMss4 to Rab proteins. Because DSS4 contains the second CysXXCys motif, it may bind  $\text{Zn}^{2+}$  in a manner similar to Mss4, possibly with Asp 20 and Asp 21 serving as the other two  $\text{Zn}^{2+}$  ligands (Fig. 2b). Other GEFs such as mSOS, Ras-GRF, and CDC25 do not possess the cysteine patterns observed in Mss4 (ref. 1). However, the possibility that these proteins bind  $\text{Zn}^{2+}$  cannot be ruled out because other amino-acid side chains can also act as  $\text{Zn}^{2+}$  ligands<sup>19</sup>.

The structures of several Ras-like proteins have been determined<sup>20-23</sup>. Although there are significant differences between them, the overall tertiary fold is conserved. The substrates of Mss4, including Rab1, Rab3, Rab8, Sec4 and Ypt1 (ref. 13), contain a highly conserved region corresponding to residues 63-77 in p21<sup>ras</sup>. Ras GTPases that do not interact with Mss4 have relatively variable sequences in this region. Based on the structure of p21<sup>ras</sup>, this region resides on a surface helix<sup>20,21</sup> and is implicated in the interactions between p21<sup>ras</sup> and its GEF, CDC25 (ref. 24). It is therefore likely that hMss4 and other GEFs contain common structural elements for the recognition of Ras proteins, despite their highly divergent sequences. □

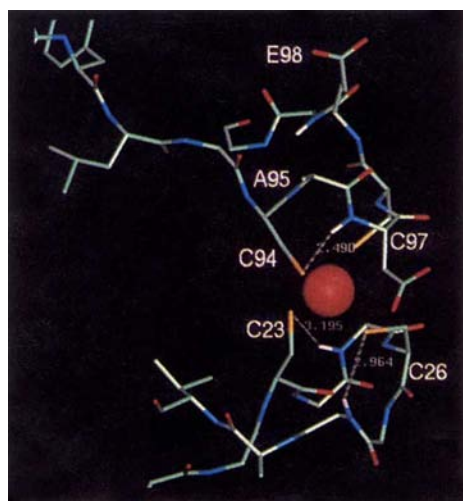


FIG. 4 The rubredoxin 'knuckle' structure observed in hMss4. The dashed lines represent hydrogen bonds; the numbers indicate the sulphur-proton distances. The zinc ion is shown as a red sphere. Generated with QUANTA.

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## Structural basis for DNA bending by the architectural transcription factor LEF-1

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LYMPHOID enhancer-binding factor (LEF-1) and the closely related T-cell factor 1 (TCF-1) are sequence-specific and cell-type-specific DNA-binding proteins that play important regulatory roles in organogenesis and thymocyte differentiation<sup>1–5</sup>. LEF-1 participates in regulation of the enhancer associated with the T cell receptor (TCR)- $\alpha$  gene by inducing a sharp bend in the DNA and facilitating interactions between Ets-1, PEBP2- $\alpha$ , and ATF/CREB transcription factors bound at sites flanking the LEF-1 site<sup>1,2,6,7</sup>. It seems that LEF-1 plays an architectural role in the assembly and function of this regulatory nucleoprotein complex<sup>7,8</sup>. LEF-1 recognizes a specific nucleotide sequence through a high-mobility-group (HMG) domain<sup>1,2</sup>. Proteins containing HMG domains bind DNA in the minor groove, bend the double helix<sup>6,9,10</sup>, and recognize four-way junctions and other irregular DNA structures<sup>9,11</sup>. Here we report the solution structure of a complex of the LEF-1 HMG domain and adjacent basic region with its cognate DNA. The structure reveals the HMG domain bound in the widened minor groove of a markedly distorted and bent double helix. The basic region binds across the narrowed major groove and contributes to DNA recognition.

The HMG domain of mouse LEF-1, corresponding to residues 296–380 of the full-length protein plus an initiator methionine residue, was complexed with a 15-base-pair oligonucleotide duplex containing the optimal binding site from the TCR- $\alpha$  gene enhancer (Fig. 1). Structures were calculated from multi-dimensional nuclear magnetic resonance (NMR) data (Table 1). LEF-1 forms an intimate complex with DNA, wrapping around and almost completely encompassing a highly distorted double helix (Fig. 2). The central framework of the protein is formed by the characteristic L-shaped arrangement of three helices and an extended region seen previously in structures of HMG-1 and HMG-D domains in the absence of DNA<sup>12–14</sup>. Helices 1 and 2 (residues 8–24 and 28–43, respectively) form one arm of the L, helix 3 (residues 46–66) and the extended N-terminal region the other. The angle between the axes of helices 2 and 3 ( $60^\circ \pm 2^\circ$ ) is more acute than in the HMG-1 and HMG-D domains<sup>12–14</sup>. Helix 3 terminates at Pro 67, after which the polypeptide kinks

TABLE 1 NMR structure statistics

|                                                      |                                        |
|------------------------------------------------------|----------------------------------------|
| (a) NMR constraints                                  |                                        |
| Protein                                              |                                        |
| Distance constraints                                 | 1,393                                  |
| Intra-residue                                        | 712                                    |
| Sequential ( $ i-j =1$ )                             | 265                                    |
| Medium-range ( $ i-j  \leq 4$ )                      | 246                                    |
| Long-range ( $ i-j  \geq 5$ )                        | 170                                    |
| Dihedral angle constraints                           | 42 $\phi$ constraints                  |
| DNA                                                  |                                        |
| Distance constraints                                 | 325                                    |
| Intra-residue                                        | 164                                    |
| Sequential                                           | 156                                    |
| Inter-strand                                         | 5                                      |
| Protein–DNA                                          |                                        |
| Distance constraints                                 | 332                                    |
| (b) Structure statistics                             |                                        |
| DIANA target function                                | 4.7 $\pm$ 1.7 (32 structures)          |
| Statistics for 12 SA structures                      |                                        |
| NOE violations                                       |                                        |
| Number $>0.2$ Å                                      | 12.3 $\pm$ 3.8                         |
| Maximum violations (Å)                               | 0.40 $\pm$ 0.06                        |
| Angle violations                                     |                                        |
| Number of violations $>5^\circ$                      | 2                                      |
| Mean constraint violation energy                     | 46.6 $\pm$ 9.9 kcal mol <sup>-1</sup>  |
| Mean AMBER energy                                    | -2,488 $\pm$ 37 kcal mol <sup>-1</sup> |
| Mean deviation from ideal covalent geometry          |                                        |
| Bond length                                          | 0.005 Å                                |
| Bond angles                                          | 1.9°                                   |
| (c) R.m.s. deviations from the mean structure        |                                        |
| Protein backbone heavy atoms                         |                                        |
| Helices                                              | 0.52 Å                                 |
| Residues Lys 4–Tyr 66                                | 0.60 Å                                 |
| Residues Met 67–Lys 85                               | 1.76 Å                                 |
| (superimposed on N, Ca, C', O)                       |                                        |
| DNA heavy atoms                                      |                                        |
| C4–G12, C19–G27                                      | 0.75 Å                                 |
| (superimposed on all DNA heavy atoms)                |                                        |
| Protein + DNA                                        |                                        |
| Helices + C4–G12, C19–G27                            | 0.75 Å                                 |
| (superimposed on N, Ca, C', O + all DNA heavy atoms) |                                        |

Interproton distance restraints were determined from nuclear Overhauser enhancement spectroscopy (NOESY) cross-peak volumes. Distances obtained from 3D <sup>13</sup>C-separated NOESY spectra<sup>24</sup> were assigned van der Waals lower bounds and upper bounds of 3.0, 4.0 and 5.0 Å. Additional upper bounds of 3.7 and 6.0 Å were used for NOEs from the <sup>15</sup>N-separated NOESY spectrum<sup>24</sup>. For protein–DNA constraints, upper bounds of 3.0, 3.5, 4.5 and 6.0 Å were used with van der Waals lower bounds. Interproton distances within DNA were calculated from a  $\tau=50$  ms NOESY spectrum at 750 MHz. The upper bound distance was increased 20% over the calculated  $r^{-6}$ -weighted distance for 2' and 2'' protons, and by 15% for all other protons. Several constraints were obtained from a  $\tau=120$  ms double half-filtered NOESY spectrum at 600 MHz, for which the upper bound was increased by 25% over the calculated distance. DNA lower bounds were set to 2.0 Å for calculated distances  $r \leq 3.0$  Å; to 2.2 Å for  $3.0 < r \leq 3.5$  Å; 2.5 Å for  $3.5 < r \leq 4.0$  Å; and 3.0 Å for  $4.0 < r \leq 6.0$  Å. Initial protein structures were calculated with the program DIANA<sup>25</sup> using the redundant dihedral angle constraint (REDAC) strategy<sup>26</sup>. Beginning with 40 randomized starting structures, three REDAC cycles were applied, followed by a single cycle using only experimental restraints and a final cycle of minimization. Initial structures were calculated using only spectroscopically unambiguous distance restraints and hydrogen-bond constraints in the known helical regions; additional NOE restraints were added during subsequent rounds of structure calculation. The 32 structures with lowest target functions were subjected to three cycles of molecular-dynamics simulated annealing (SA) using the AMBER 4.1 package and all-atom force field<sup>27</sup>, modified to reduce charges to 20%. Additional sets of non-NMR restraints were used in the initial stages of SA: helical hydrogen bond restraints on protein residues 11–24, 31–43 and 48–66, Watson–Crick hydrogen bond constraints on DNA, and distance constraints between sugar and base heavy atoms to maintain B-form DNA. The first cycle involved docking of the DIANA-refined protein structures, placed about 50 Å away from the binding site, onto B-form DNA. The two subsequent SA cycles began from the end points of the preceding cycles. In each cycle, the temperature was increased from 0 to 600 K over 6 ps, then cooled to 0 K over 14 ps with a temperature relaxation parameter of  $1.2 \text{ ps}^{-1}$ . The force constant for violation of the distance restraints was 20 kcal mol<sup>-1</sup> Å<sup>-2</sup>. During the first SA cycle, the experimental protein–DNA restraints were slowly turned on while the distance constraints initially imposed to keep the DNA in B-form were slowly turned off for the central 9 bp. Of the 32 DIANA starting structures, 29 led to acceptable docked structures and were subjected to subsequent SA cycles. During the second cycle, the remaining B-DNA constraints (on the outer 6 bp) and the hydrogen bond constraints in the protein helices were also turned off. The final SA cycle included only the NMR-based restraints, the Watson–Crick hydrogen bond constraints, and the AMBER potential functions. Of the 29 acceptable structures, 12 with no constraint violations greater than 0.5 Å were minimized for an additional 200 steps with the same constraints as for the final SA cycle.

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sharply and extends away from the core of the protein, making contacts predominantly with DNA. Indeed, the carboxy-terminal region beyond Arg 72 contacts only DNA.

An extensive hydrophobic core is enclosed by helices 1 and 2 and the amino-terminal region of helix 3 (Fig. 2b). The aromatic side chains of Tyr 12, Trp 40, Tyr 51, and Tyr 52 are packed edge-to-face in the junction between the three helices; Ala 8, Leu 11, Leu 43 and Leu 54 complete this region of the hydrophobic core. The core is extended along the interface between helix 1 and 2 by Met 13, Met 16, Val 20 and Val 21 in helix 1, and by Leu 36, Ile 35 and Ile 32 in helix 2. There is a small hydrophobic cluster involving the side chains of Ile 2, Tyr 66 and Trp 69 at the end of the third helix.

The DNA duplex binds to the concave surface of the LEF-1 domain and is bent severely towards the major groove (Fig. 2c). Cross-strand nuclear Overhauser enhancement (NOE) connectivities indicate retention of Watson-Crick base pairs. A wide and very shallow minor groove is formed between C5 · G26 and G9 · C22 that can accommodate helices 1 and 2 of LEF-1. The average minor groove width in this region is 11 Å with an average depth of ~1 Å; it is thus comparable in width to the minor groove of A-DNA but is even shallower. From A10 · T21 to C13 · G18 the minor groove becomes narrower (average width 5 Å) and deeper (average 4.6 Å). Bending and opening of the minor groove is accompanied by substantial narrowing (to <6 Å on average, cf. 11.7 Å for B-DNA) and deepening (to >7 Å on average) of the major groove from C5 · G26 to T8 · A23. The major groove subsequently becomes more like B-DNA in width although it remains rather deep. Bending occurs throughout the 9 base-pair (bp) recognition element, with an average total curvature summed over the 15-bp path of  $117^\circ \pm 10^\circ$  in the 12 NMR structures. The curvature is not uniform but is most pronounced at the C5 · G26 : T6 · A25 step and the T7 · A24 : T8 · A23 step. The double helix is significantly underwound at the C5 · G26 : T6 · A25 and T6 · A25 : T7 · A24 steps, with average

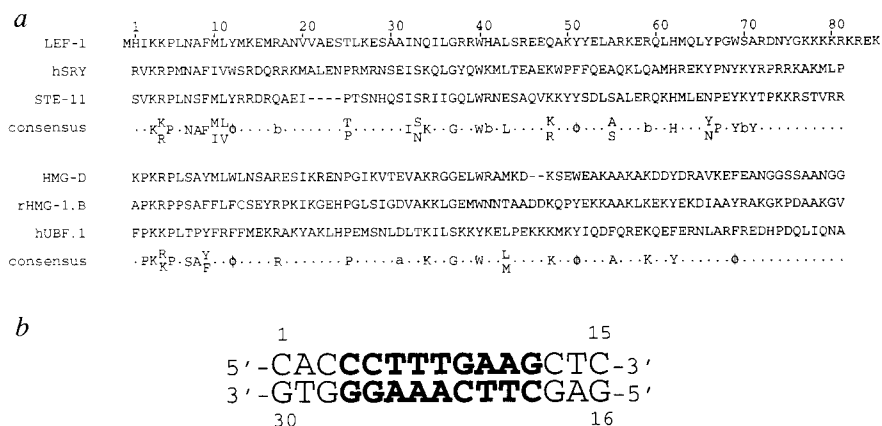
twist angles of  $19.1^\circ \pm 2.8^\circ$  and  $24.3^\circ \pm 2.8^\circ$ , respectively, compared with  $31.7^\circ$  twist for regular B-DNA.

The LEF-1 HMG domain makes extensive and continuous contacts in the DNA minor groove from C4 · G27 to A11 · T20, encompassing the entire region implicated in binding by chemical footprinting<sup>15,16</sup> and mutagenesis<sup>3,6</sup>. The protein-DNA contacts are shown schematically in Fig. 3b. The most extensive interactions involve hydrophobic residues exposed on the concave surface formed by helices 1 and 2 and the extended N-terminal region. Further contacts are made by side chains from the C-terminal region where it crosses the minor groove (Fig. 3a). In particular, Tyr 75 is inserted into the narrowed region of the minor groove at the 5' end of the TCR-α recognition element and appears to play a key role in DNA bending. The aromatic ring is in close contact with the ribose rings of G12 and T21 and the bases of G12, T20 and T21 (Fig. 3d). Interestingly, replacement of the G12 · C19 base pair by T · A has little effect on binding affinity but significantly diminishes the bending angle as measured by circular permutation assays (K.G. and R.G., unpublished observations). Polar side chains tend to contact the sugar-phosphate backbone rather than hydrogen bonding to bases. Exceptions are Asn 7 and Glu 28, which are located in the minor groove in close proximity to the 2-amino groups of G9 and G26/G27, respectively.

The side chain of Met 10 is partially inserted into the base stack between A23 and A24 (Fig. 3c), disrupting base stacking but not base pairing. Opening of the bases on the minor groove side leads to a large negative roll angle ( $-52^\circ \pm 6^\circ$ ) and an increase in rise to  $4.8 \pm 0.3$  Å at this step. Additional stabilizing contacts in this region are made by the side chain of Met 13, which is in close proximity to the A24 base, and the aromatic ring of Phe 9, which packs against the hydrophobic surface of the T8 ribose. These interactions probably play a major role in DNA bending through stabilization of the widened minor groove. An A · T to T · A transversion at position 24 results in

FIG. 1 a, Sequence alignments of representative members of the two subfamilies of HMG domain proteins (from ref. 8). Consensus sequences are shown for each subfamily. The exact numbering scheme of the murine LEF-1 HMG domain used here is indicated. The sequence differs from wild-type in that it retains the N-terminal initiator methionine, and Cys 24 has been mutated to serine to avoid potential problems during long-term NMR experiments. Neither modification has a discernible effect on the DNA binding affinity. b, Sequence and numbering scheme of the oligonucleotide duplex used for complex formation. The nine base pairs involved in LEF-1 binding are indicated in bold type.

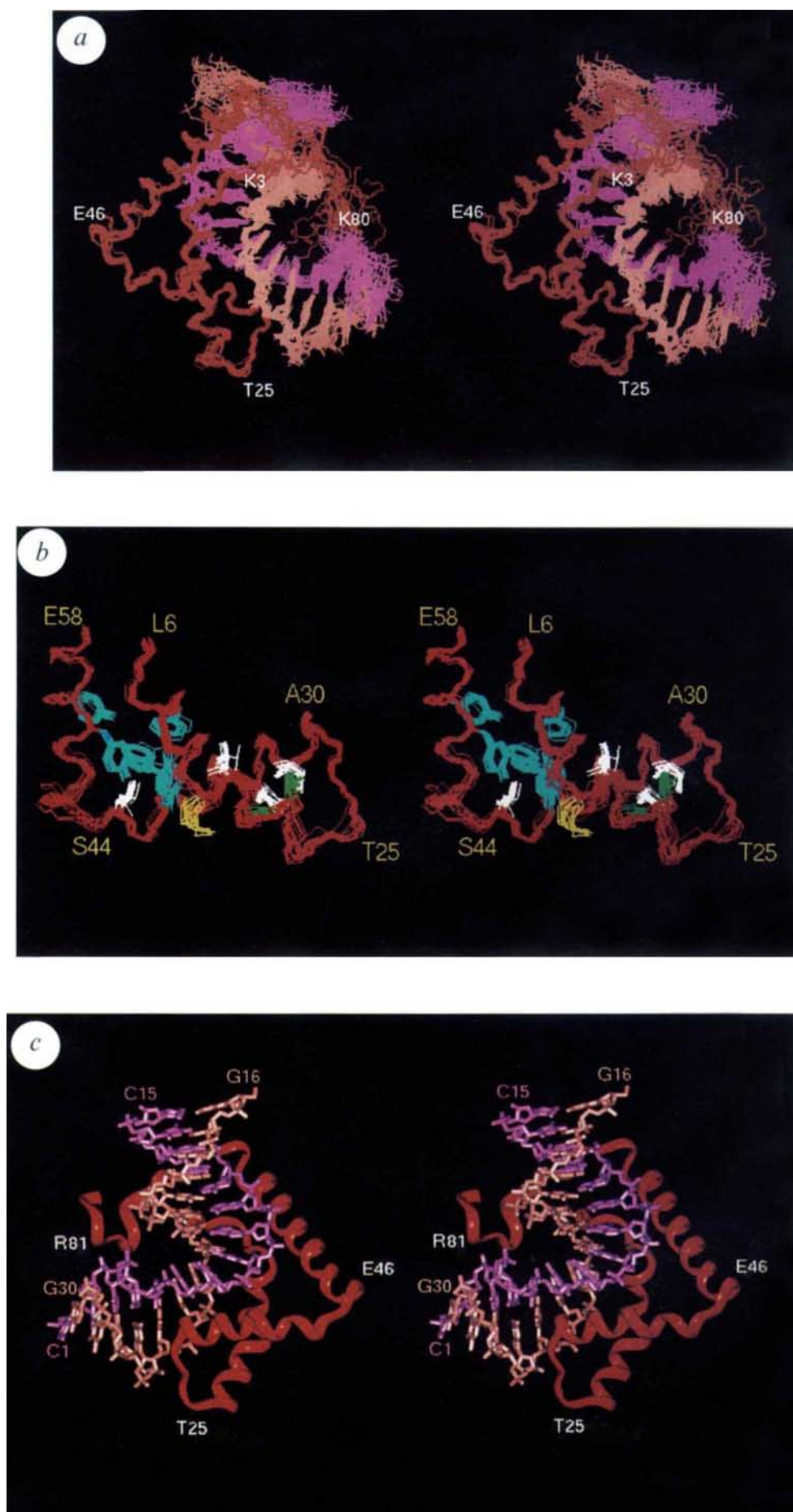
**METHODS.** The LEF-1 HMG domain was expressed in *Escherichia coli* and labelled uniformly with  $^{13}\text{C}$  and/or  $^{15}\text{N}$ . The purified protein was shown to have the correct sequence by ion-spray mass spectrometry and was fully functional in DNA binding. DNA was synthesized by the phosphoramidite method and purified by reverse-phase HPLC on a C-18 column (0.1 M triethylamine-acetic acid buffer, pH 6.5, eluted with an acetonitrile/water gradient) before removal of the dimethoxytrityl protecting groups. After deprotection, the duplex was formed by mixing equimolar amounts of each oligonucleotide and annealed by slow cooling from  $75^\circ\text{C}$  over 12 hours. HiTrap Q anion exchange was used for final purification. The complex was formed by titration of protein (in 10 mM KCl) into the DNA duplex (in 200 mM KCl, pH 5.5), using changes in the DNA imino proton spectrum to indicate 1:1 stoichiometry, and was desalted and concentrated using a Centricon to 1–2 mM in 10 mM KCl, pH 6.7, for NMR. Spectra were acquired at 303 K using Bruker DMX750, AMX600 and AMX500 spectrometers and processed with Felix 2.3 (Biosym Technologies). Protein resonance assignments (to be described elsewhere) were obtained by using extensive 2D and 3D NMR experiments ( $^{15}\text{N}$ -separated total correlation spectroscopy (TOCSY),  $^{15}\text{N}$ -separated NOESY,



constant time-HNCA, HNCACB, CBCA(CO)NH, HBHA(CO)NH, C(CO)NH-TOCSY, H(CO)NH-TOCSY, HCCH-COSY, HCCH-TOCSY,  $^{13}\text{C}$  heteronuclear multiple bond correlation (HMBC) and aromatic  $^{13}\text{C}$ -H correlation spectroscopy). Distance restraints were derived from  $^{13}\text{C}$ -separated NOESY spectra (mixing times  $\tau = 60$  and 120 ms), a  $^{15}\text{N}$ -separated NOESY spectrum<sup>27</sup> ( $\tau = 120$  ms) and 750 MHz  $^1\text{H}$  homonuclear NOESY spectra ( $\tau = 50$  and 120 ms). Backbone  $\phi$  dihedral angle constraints were obtained from  $^3J_{\text{HN,H}\alpha}$  coupling constants measured in an HNCA-J experiment<sup>28</sup>. DNA resonances were assigned from 2D [ $^{15}\text{N}$ ,  $^{13}\text{C}$ ]-double half-filtered NOESY and [ $^{15}\text{N}$ ,  $^{13}\text{C}$ ]-half-filtered TOCSY spectra<sup>29</sup>. DNA distance restraints were derived from double half-filtered NOESY and  $^{13}\text{C}$ -decoupled homonuclear  $^1\text{H}$ - $^1\text{H}$  NOESY spectra at 750 MHz ( $\tau = 50$  and 120 ms). NOEs between the protein and the DNA were identified specifically in a 3D  $^{13}\text{C}(\omega_1, \omega_2)$ -edited,  $^{13}\text{C}(\omega_3)$ -selected NOESY experiment ( $\tau = 120$  ms) (to be reported elsewhere).



FIG. 2 *a*, Stereo view showing the best-fit superimposition of 12 structures of the LEF-1 HMG domain complexed with DNA. The structures were superimposed on all backbone heavy atoms of residues 6–66 and all heavy atoms of base pairs 4–12. The backbone N, C $\alpha$  and C' atoms of the protein are shown in red. All non-hydrogen atoms of the DNA are shown, coloured bright pink for nucleotides C1–C15, and salmon pink for G16–G30. The C1 · G30 base pair is at the lower right and the C15 · G16 base pair is at the top centre. *b*, Stereo view showing the packing interactions in the hydrophobic core of the LEF-1 HMG domain. The structures were superimposed on all backbone heavy atoms of residues 6–66 of the protein. The polypeptide backbone is shown in red, the aromatic side chains of Phe 9, Tyr 12, Trp 40, Tyr 51 and Tyr 52 in cyan, the side chains of Val 20 and Val 21 in green, Ile 32, Ile 35, Leu 36 and Leu 43 in white, and Met 16 in yellow. *c*, Stereo view of the complex from the opposite side of the DNA from that shown in *a*. The HMG domain is represented by the red tube drawn through the backbone N, C $\alpha$  and C' atoms. The pronounced bend in the DNA ( $117^\circ \pm 10^\circ$ ) is evident in this view. The DNA curvature and helical parameters were calculated with the program CURVES<sup>30</sup>.





a severe loss of binding affinity<sup>3</sup>. Moreover, transversion of A25 to thymine substantially decreases the bending angle but has little effect on binding affinity (K.G. and R.G., unpublished observations). The side chain of Ile 68 in the sex-determining factor SRY, corresponding to Met 10 of LEF-1, also penetrates partly into the base stack and is required to induce specific DNA

bending but is not required for binding to an intrinsically bent four-way junction<sup>17</sup>.

In addition to the minor groove interactions, residues from the highly basic C-terminal region bind across the narrowed major groove, making contacts primarily with the sugar-phosphate backbone (Figs 2c and 3a). Mutation of Lys 79 and Lys 80 or

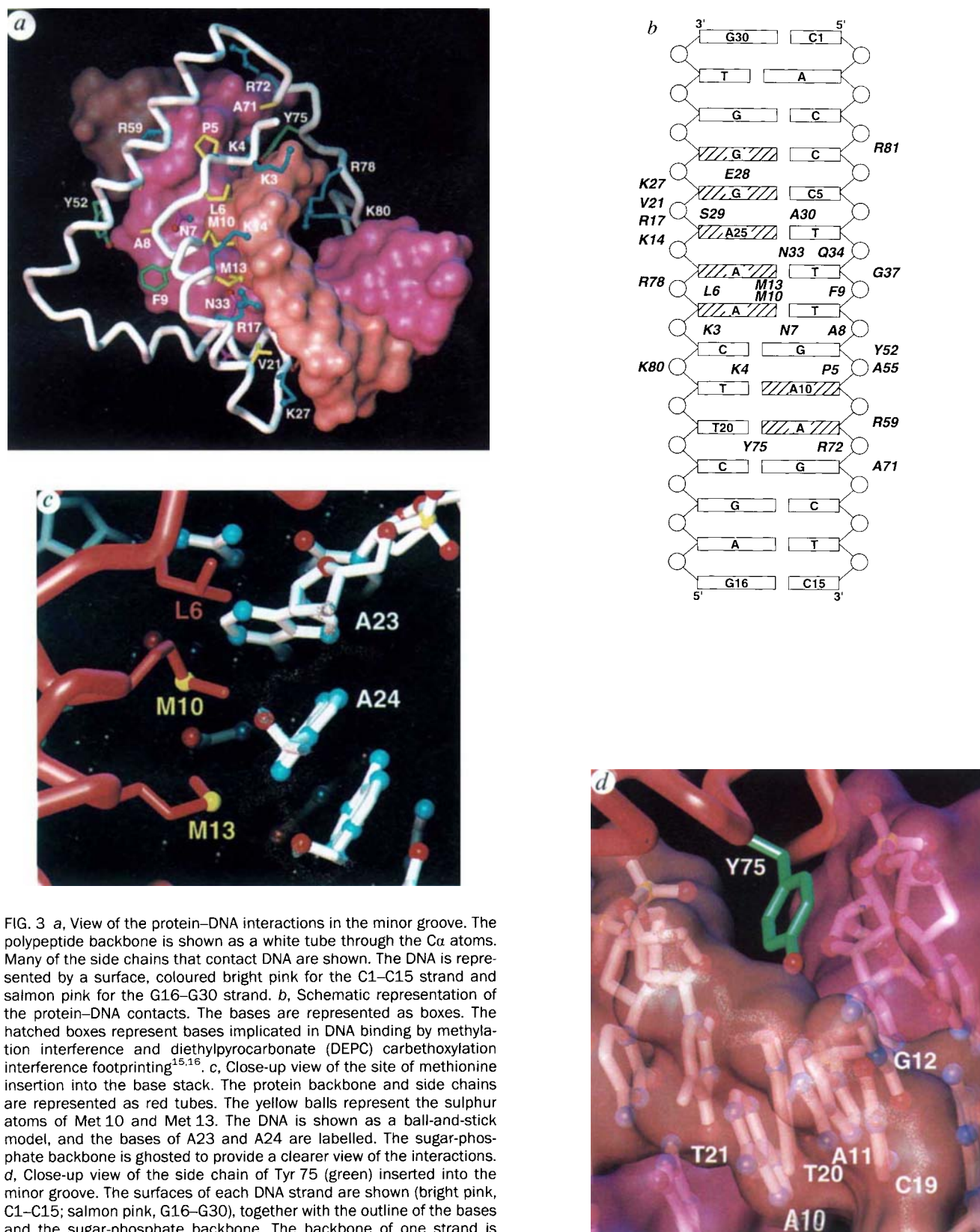


FIG. 3 **a**, View of the protein-DNA interactions in the minor groove. The polypeptide backbone is shown as a white tube through the C $\alpha$  atoms. Many of the side chains that contact DNA are shown. The DNA is represented by a surface, coloured bright pink for the C1-C15 strand and salmon pink for the G16-G30 strand. **b**, Schematic representation of the protein-DNA contacts. The bases are represented as boxes. The hatched boxes represent bases implicated in DNA binding by methylation interference and diethylpyrocarbonate (DEPC) carbethoxylation interference footprinting<sup>15,16</sup>. **c**, Close-up view of the site of methionine insertion into the base stack. The protein backbone and side chains are represented as red tubes. The yellow balls represent the sulphur atoms of Met 10 and Met 13. The DNA is shown as a ball-and-stick model, and the bases of A23 and A24 are labelled. The sugar-phosphate backbone is ghosted to provide a clearer view of the interactions. **d**, Close-up view of the side chain of Tyr 75 (green) inserted into the minor groove. The surfaces of each DNA strand are shown (bright pink, C1-C15; salmon pink, G16-G30), together with the outline of the bases and the sugar-phosphate backbone. The backbone of one strand is ghosted for clarity.

truncation at Lys 77, Lys 78 or Lys 80 significantly impairs DNA binding (refs. 15, 18, 19, and results not shown), thus delineating the minimal domain for high-affinity binding as His 1–Arg 81. A polypeptide containing the LEF-1 HMG domain plus the basic residues bends DNA in circular permutation assays by  $\sim 130^\circ$  (ref. 6), but only by  $50^\circ$  when truncated at Lys 77 (ref. 19). We conclude that the basic region C-terminal to the HMG domain contributes to both DNA recognition and bending. Sequence variation in this region may, in part, account for the difference in the previously estimated angle of DNA bending by LEF-1 ( $130^\circ$ ) and the mammalian testis-determining factor SRY ( $83^\circ$ )<sup>6,9</sup>.

The NMR structure forms a basis for understanding mutagenesis experiments on LEF-1 and provides insights into the likely function of several conserved amino acids. Mutation of residues 3, 4, 6, 10, 13, 37, 40, 51, 79 and 80 impairs or abrogates DNA binding (ref. 15 and unpublished observations). The majority of these mutations involve DNA contact residues; Trp 40 and Tyr 51 form the hydrophobic core at the junction of the three helices and are probably essential for stabilization of the folded structure. Several DNA contact residues are highly conserved (see ref. 8 and Fig. 1 for sequence alignments). Basic side chains are favoured at positions 3, 4, 17, 34 (Gln in LEF-1) and 59, which interact predominantly with the DNA backbone in the LEF-1 complex. An aromatic residue is strongly conserved at position 9, presumably to retain optimal ribose packing, and a bulky hydrophobic side chain is favoured at the site of insertion into the base stack (position 10). There is a marked preference for a small polar side chain at position 7, where there are base contacts in the LEF-1 complex. Finally, small side chains are conserved at positions 8, 37 and 55, with a strong preference for Ala, Gly and Ala, respectively. All three sites are in close proximity to the sugar-phosphate backbone and substitution by residues with bulky side chains would be expected to interfere with DNA binding. Indeed, replacement of Gly 37 by Ala in LEF-1 and by Arg in SRY impairs (LEF-1) or abrogates (SRY) DNA binding (ref. 20, and unpublished work).

HMG-domain proteins can be classified into two subfamilies<sup>8</sup>. Members of one subfamily, which includes the abundant HMG-1 and HMG-2, have multiple HMG domains, bind DNA with little or no specificity and are found in all cell types. Members of the other subfamily, which includes the lymphoid enhancer-binding factors LEF-1 and TCF-1 (refs 1–3), contain a single HMG domain, bind DNA sequence-specifically and are

expressed in few cell types. Comparison of the structure of the LEF-1 HMG domain bound to DNA with structures of the unbound HMG-1 and HMG-D domains<sup>12–14</sup> leads to a highly plausible structural model to explain the different DNA-binding activities of the two subfamilies. In LEF-1, helix 3 is significantly shorter than in the HMG-1 and HMG-D domains, being limited in length by Pro 67, which induces a pronounced kink in the polypeptide chain and allows the C-terminal region to make extensive contacts with DNA, thereby greatly extending the binding surface. Contacts include insertion of Tyr 75 deep into the minor groove and interactions of the basic amino acids adjacent to the HMG domain with the sugar-phosphate backbone. Pro 67 is invariant in HMG-domain proteins that bind DNA with sequence-specificity but is absent from the subfamily that exhibits little or no specificity in binding DNA (see ref. 8 for sequences). Further, many members of the HMG-1 subfamily lack the highly basic region immediately adjacent to the HMG domain. However, these proteins retain the characteristic L-shaped fold and many of the DNA contact residues in the HMG core, which explains their ability to bind non-specifically but with high affinity to four-way junctions and other intrinsically bent DNA structures. Thus it appears that additional protein–DNA interactions, facilitated by the Pro 67-induced kink in the HMG domain and by the adjacent basic region, are important for high-affinity DNA binding and induction of DNA bending.

The structure of the LEF-1 HMG domain complexed with DNA provides new insights into the molecular basis for minor groove binding and protein-induced DNA bending. The LEF-1 HMG domain, the TATA-binding protein<sup>21,22</sup>, and the purine repressor<sup>23</sup> induce large angle bends in DNA by a common mechanism, involving opening of the minor groove and partial insertion of one or more hydrophobic side chains into the base stack from the minor groove side. In each case the DNA is bent towards the major groove and away from the bound protein. DNA bending induced by proteins with single or multiple HMG domains appears to play an architectural role in the assembly of higher-order nucleoprotein complexes by juxtaposing non-adjacent factor-binding sites and/or positioning activation domains for interaction with other regulatory proteins<sup>8</sup>.

*Note added in proof:* After submission of this paper, the structure of a DNA complex of human SRY has been reported<sup>31</sup>. The protein–DNA interactions observed for SRY are generally similar to those of LEF-1, but differ in several details, mainly at the C terminus. □

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# A novel thermostable polymerase for DNA sequencing

Michael A. Reeve and Carl W. Fuller

**Using recent findings by Tabor and Richardson, a novel thermostable DNA polymerase that generates sequence data comparable with T7 Sequenase DNA polymerase has been developed for improved base calling and longer reads.**

THERMO Sequenase is a novel thermostable DNA polymerase specifically engineered for DNA sequencing by Amersham using a recent finding of Tabor and Richardson<sup>1</sup>. As with T7 Sequenase DNA polymerase, Thermo Sequenase generates uniform (and therefore easy-to-read) sequence data. In addition, the thermostability of this polymerase makes cycle sequencing possible — thereby combining base calling accuracy comparable with T7 Sequenase DNA polymerase with the sensitivity and reliability of cycle sequencing.

When sequencing DNA by the chain-termination method<sup>2</sup>, a DNA polymerase is used to synthesize DNA in the presence of a mixture of deoxynucleoside triphosphates (dNTPs) and a dideoxynucleoside triphosphate (ddNTP). Most members of the DNA polymerase I-type family of DNA polymerases catalyse the addition of ddNTPs to the growing DNA chain at about 0.02–0.1 per cent the rate of dNTPs. An exception is bacteriophage T7 DNA polymerase, which incorporates ddNTPs more efficiently; at about 20 per cent the rate of dNTPs.

In order to determine the structural basis for the above discrimination against ddNTPs, Tabor and Richardson<sup>1</sup> prepared a number of hybrid polymerases. These hybrids were constructed by individually replacing conserved sequence regions of T7 DNA polymerase with the corresponding sequence from *E. coli* DNA polymerase I. The opposite replacements were also made. The hybrid polymerases were assayed for activity in the absence and presence of a concentration of ddTTP, which strongly inhibits T7 polymerase but not *E. coli* DNA polymerase I. By analysis of a large number of such hybrids, they found that the presence of a single hydroxyl group in the polypeptide chain can change the efficiency of ddNTP usage by more than 1,000-fold<sup>1</sup>.

Specifically, replacing tyrosine 526 of T7 DNA polymerase with phenylalanine decreases the efficiency of ddNTP usage more than

2,000-fold<sup>1</sup>. Replacing the analogous amino acid in *E. coli* DNA polymerase I (phenylalanine 762) or *Taq* DNA polymerase (phenylalanine 667) with tyrosine increases the efficiency of ddNTP usage 250- to 8,000-fold<sup>1</sup>. These modified polymerases can be used for chain-termination sequencing, and when used appropriately, they can produce more accurate and reliable sequence data.

## Thermo Sequenase

Many DNA polymerases, such as *E. coli* DNA polymerase I, have 5'–3' exonuclease activity. This activity can be undesirable for many DNA sequencing methods because it can hydrolyse nucleotides from the 5' end of the sequencing primer, giving rise to spurious bands upon gel electrophoresis<sup>3</sup>. The amino-terminal region of the *E. coli* DNA polymerase I protein is responsible for its 5'–3' exonuclease activity. As shown by Klenow<sup>4</sup>, this region can be removed by limited proteolysis. The N-terminal region can also be removed by deletion or mutation of the corresponding portion of the gene<sup>5</sup>, leaving a fully active polymerase. A similar

approach has been used to construct thermostable polymerases lacking exonuclease activity<sup>6,7</sup>. In order to construct this new DNA polymerase, we have combined the genetic manipulation required to produce an exonuclease-free thermostable polymerase with the phenylalanine-to-tyrosine mutation to remove discrimination against ddNTPs.

DNA polymerases, like all enzymes, are catalysts that can accelerate reactions in both directions. When a polymerase is incubated with DNA and pyrophosphate, it will catalyse pyrophosphorolysis of the DNA, removing mononucleotides from the DNA chain to form dNTPs. Thermo Sequenase, like other DNA polymerases that efficiently incorporate ddNTPs, can remove 3'-terminal dideoxymononucleotides from DNA by pyrophosphorolysis, even under typical sequencing reaction conditions. This occurs faster at some termini than others, resulting in weak sequence bands or peaks.

Fortunately, pyrophosphorolysis can be prevented by adding inorganic pyrophosphatase, an enzyme that hydrolyses pyrophosphate. We have purified a thermostable inorganic pyrophosphatase from a

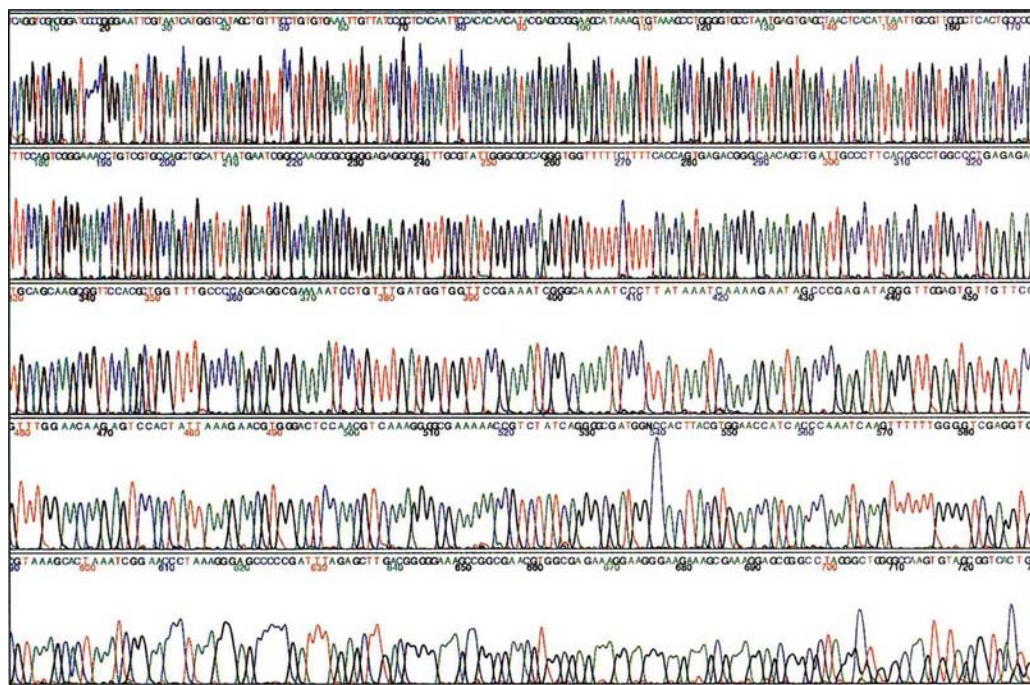


FIG. 1 Thermo Sequenase dye-primer cycle sequencing. M13 template preparation and sequencing reactions were performed on the DNA Labstation 625. Samples were run on an Applied Biosystems 373 Stretch sequencing instrument. The template for this sequence is M13mp8.

thermophilic archaeobacterium, which eliminates sequencing problems caused by pyrophosphorylation. We add this thermostable inorganic pyrophosphatase to all Thermo Sequenase cycle sequencing reactions.

## Cycle sequencing

During DNA sequencing, DNA is synthesized starting from a specific priming site and ending with the incorporation of a chain-terminating nucleotide, such as a ddNTP (ref. 2). The resulting DNA fragments are then separated by size using electrophoresis through a polyacrylamide gel matrix. Cycle sequencing<sup>8-12</sup> uses repeated cycles of thermal denaturation, annealing and extension with termination to increase the amount of sequencing products by effectively reusing the template DNA. Cycle sequencing is ideally suited for applications using low amounts of template or a detection system with limited sensitivity. For this reason, cycle sequencing is commonplace whenever fluorescent labelling is used.

## DNA sequencing methods

We have investigated the use of Thermo Sequenase in many different DNA sequencing methods, employing either fluorescent or radioactive detection. For fluorescent sequencing, we have incorporated Thermo Sequenase into four-dye primer, one-dye primer and dye-terminator, cycle-sequencing protocols. For radioactive sequencing, we have used Thermo Sequenase with both radiolabelled primers and radiolabelled nucleotides for cycle sequencing. Our results point to the following conclusions.

*Taq* DNA polymerase, as well as other thermostable polymerases used in cycle sequencing, tend to incorporate dideoxynucleotides unevenly, generating highly variable peak heights. Peaks with uneven heights can be difficult to analyse accurately (especially when the sequence has high background noise). Thermo Sequenase incorporates ddNTPs at rates that are independent of neighbouring sequence, resulting in uniform peak heights. Increased base calling accuracy is therefore possible and most noticeable at longer read lengths — allowing more data to be obtained from a given electrophoretic run. An example showing fluorescent dye-primer cycle sequencing with Thermo Sequenase is given in Fig. 1.

Thermo Sequenase can facilitate the automation of dye-terminator cycle sequencing reactions. The reduced discrimination against ddNTPs that has been engineered into this DNA polymerase also manifested itself in the greater acceptance of fluorescent-dye terminators as substrates when compared with other thermostable DNA polymerases. Lower concentrations of

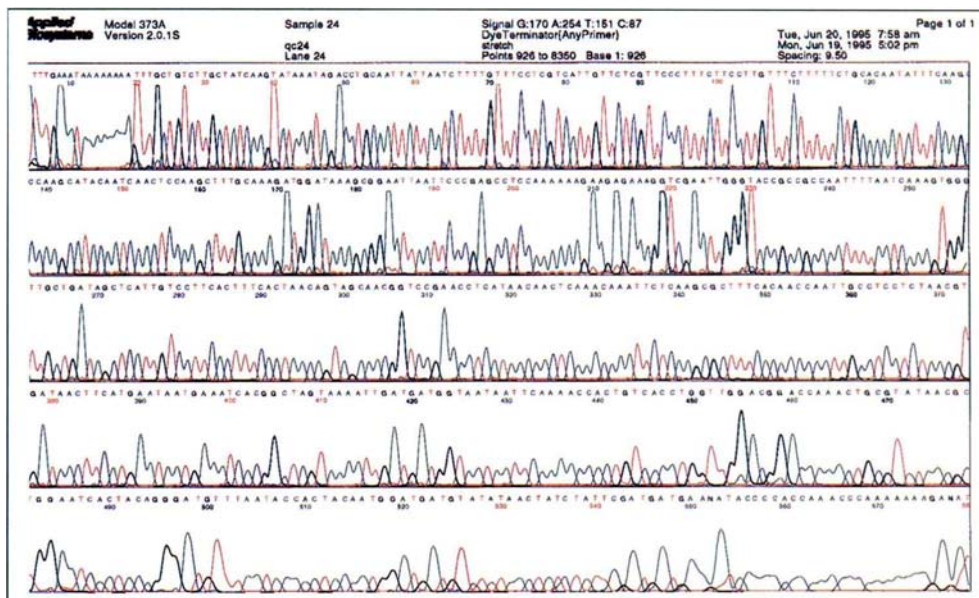


FIG. 2 Thermo Sequenase dye-terminator cycle sequencing with dITP. Plasmid template preparation, sequencing reactions and dye-terminator removal were performed on the DNA Labstation 625. Samples were run on an Applied Biosystems 373 Stretch sequencing instrument. The template for this sequence is pDM20.

fluorescent-dye terminators can therefore be used in the sequencing reaction, leaving far less residual unincorporated dye-terminator to remove from reaction products before loading the sequencing gel. Removal of residual unincorporated dye-terminator from cycle sequencing reactions is therefore a much simpler task, whether performed manually or automatically.

For the automated removal of residual unincorporated dye-terminators, we have made use of a proprietary enzymatic, precipitation method on the Vistra DNA Labstation 625. Automation of dye-terminator cycle sequencing reactions and subsequent removal of residual unincorporated dye-terminators has been demonstrated. With the addition of an automated template preparation, complete 'cells-to-gels' automation is possible. An example showing automated dye-terminator cycle sequencing with Thermo Sequenase is given in Fig. 2.

Thermo Sequenase is also useful for cycle sequencing with radioactive labels. Uneven band intensity patterns on autoradiograms can be difficult to analyse accurately (especially when the sequence is faint or the bands are closely spaced). The combination of even band intensities and cycle sequencing with Thermo Sequenase leads to improved base calling accuracy even when sequencing very small amounts of template. Using two-step, cycle-sequencing protocols<sup>3</sup>, sequences can be obtained with as little as 5–10 fmol of template DNA.

## Future prospects

We have found that Thermo Sequenase gives improved base calling with both fluorescent and radioactive cycle sequencing. In addition, we have found that this new DNA polymerase can facilitate the automation of dye-terminator cycle sequencing reactions. These results

have also been replicated at major genome centres in North America and Europe.

Amersham are currently bringing the above properties of Thermo Sequenase to market with a range of DNA sequencing kits for manual and automated sequencing reactions with fluorescent and radioactive labelling. Our aim has been to supply these kits in the most convenient form. We have carried out extensive testing of premixed and stabilized formats. Wherever possible these kits are stored unfrozen, with all components premixed for minimal pipetting.

Amersham intend to continue the study of this remarkable thermostable polymerase and to extend the range of kits offered in both manual and automated applications.

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# Ribonucleic recap

Featuring molecular biology, this week's edition offers concise information on new tools for the laboratory, such as a new thermostable reverse transcriptase and gene-identification and point mutation screening kits.

Available from Johnson & Johnson Clinical Diagnostics is the company's self-contained Pouch for PCR reagents, which is designed to **capture and concentrate DNA and to remove inhibitory substances** (*Reader Service No. 101*). The manufacturer states that it reduces the time required for sample preparation to less than 30 min. Amplification efficiency is said to increase with automatic hot starts, as a consequence results are produced within an hour of loading the prepared sample pouch onto the system. The pouch design is said to allow multiplexing and the ability to perform multiple detections from a single specimen.

The Bobby trolley from Radleys is a **multipurpose mobile storage unit** for use in the research laboratory (*Reader Service No. 102*). It is designed to work in areas where there is a need to have instruments and equipment read-

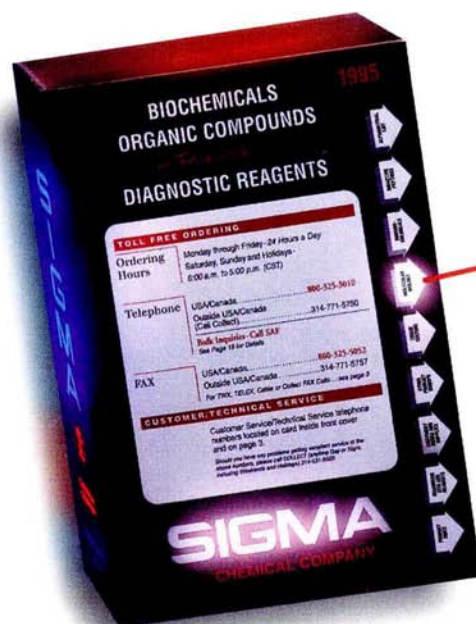


The Bobby instrument trolley from Radleys

ily at hand. The trolley has a unique combination of pivoting trays, shelves and handy recesses. The top work surface measures 41 cm × 43 cm. It is manufactured from

injection-moulded plastic and mounted on heavy duty rubber castors for low-noise movement.

Stratagene's RAP-PCR kits have been introduced for **identifying selectively expressed genes** (*Reader Service No. 103*). The RNA arbitrarily primed polymerase chain reaction (RAP-PCR) is designed to provide researchers with a simple and reproducible alternative to differential display techniques. This method uses an 18-base arbitrary primer in both the first-strand cDNA synthesis reaction and PCR. The PCR consists of a single round of low-stringency amplification followed by multiple rounds with higher stringency. Structuring the PCR in this manner is said to



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minimize the amplification of genomic (gDNA), so that the signal is derived primarily from RNA, not from gDNA. The differential display techniques, which use a 10-base oligomer and low-stringency annealing throughout the procedure, have the potential to amplify gDNA, which can complicate the RAP pattern and reduce information derived from RNA. This method is said to increase the likelihood of amplifying within the coding region of mRNA because the arbitrary primers can anneal throughout the entire mRNA. In contrast, differential display is limited to amplifying regions 100–500 bases upstream of the poly(A) tract in mRNA so the coding regions might not be amplified. The larger 18-oligomer primers allow the incorporation of restriction sites into primers for custom primer design — the company offers three different primer sets.

Grant Instruments can supply an **environmental control system** to meet various analytical requirements, including heating or cooling laboratory samples under wet and dry conditions (*Reader Service No. 104*). The company's stirred baths are designed to perform powerful stirring and to ensure temperature uniformity. They are available with digital or analogue controls. Universal and unstirred water baths are for



**Grant's environmental control systems.**

routine, controlled-temperature procedures, and operate in the ambient range from 5 °C to 99 °C. Low temperature baths and circulators operate on 'ozone-friendly' refrigerant and are designed to provide precision controlled cooling for many types of scientific apparatus. The recirculating chillers are said to operate at temperatures as low as –10 °C and to provide constant pressure

flow at a precise repeatable temperature. Also offered are digital block heaters, suitable for a wide variety of applications within the temperature range from 5 °C to 150 °C. Interchangeable blocks are available to accommodate many different shapes and sizes of vessel.

Ambion's new **MisMatch Detect point mutation screening kit** provides a simple rapid method for detecting dispersed point mutations in a non-isotopic format (*Reader Service No. 105*). In the non-isotopic RNase cleavage assay, the substrates for RNase digestion are RNA/RNA duplexes made by hybridizing complementary, wild-type and experimental transcripts. The RNase cleavage products are separated by electrophoresis on native agarose gels containing ethidium bromide and assessed under ultraviolet light. The cleavage products in the experimental samples are compared with those from a no-mismatch control sample also treated with RNase. Samples are scored as positive for mutations based on the presence of smaller fragments present only in the experimental lanes. Target regions of at least 500 bases can be efficiently screened in a single step. The whole procedure, including data analysis, can be completed in less than a day.

## Doris Dixon

- Presently researching protein expression and DNA/RNA isolation and purification systems.
- Published in *Gene*, *Journal of Immunology*, and *Molecular Biology of the Cell*.
- Presented abstracts at Symposium for Protein Society and ASBMB/DBC-ACS Joint Meeting.
- Prior to Sigma, worked four years in the Department of Molecular and Cellular Biology at a major pharmaceutical company.
- Also worked ten years in academia at the University of Missouri, the University of Texas and the Wadsworth Research Institute at the New York State Department of Health.



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## PRODUCT REVIEW

Now available from Promega is the company's reverse transcription system, which utilizes **AMV reverse transcriptase** to **synthesize single-strand cDNA** from total or poly(A)<sup>+</sup> RNA (*Reader Service No. 106*). The system provides reagents to efficiently reverse transcribe RNA in 15 min and contains sufficient reagents for 100 reactions, processing up to 1.0 µg of RNA per reaction. A 1.2-kb RNA transcript with a poly(A) tail is provided as a control template for the cDNA synthesis.

Max is a new **rechargeable, portable pipette controller** from CamLab with a built-in memory function, offering faster and more accurate dispensing (*Reader Service No. 107*). Once the unit is set, it will either aspirate and stop at the desired volume automatically or function as a step pipettor, dispensing the set volume until the pipette is empty. There is a three-position switch to determine the function, with trigger-style buttons to aspirate and dispense. It will accept any standard glass or plastic pipette up to 100 ml (outer diameter 2–10 mm), and incorporates an autoclavable pipette holder. A replaceable hydrophobic filter prevents overfilling and protects against contamination. A full, four-hour charge on the NiCad batteries gives a full day's pipetting, and the automatic sleep function prolongs battery life by turning off the power when the controller is idle for 15 min.



CamLab's portable pipette controller.

The new Progene **thermal cycler** from Techne has just been released for use in molecular biology and diagnostic laboratories and is suitable for use with diagnostic kits (*Reader Service No. 108*). It is said to include user-friendly software that can accommodate the latest PCR protocols such as 'Touchdown', 'RAPD' and 'Long PCR'. The manufacturer states that a typical 25-cycle programme can be completed in about 1 h with a cooling/heating rate of better than 2–3 °C s<sup>-1</sup>. This thermal cycler is available in two models for 20 × 0.5 ml microcentrifuge tubes and 25 × 0.2 microtube/Costar 5 × 5 Thermowell plates. The cycler operates over the tempera-

ture range from 4 °C to 99 °C using Peltier technology and is stated to have a block uniformity of better than ±0.5 °C for both internal and external control and calibration. Other features included as standard are interchangeable blocks, automatic restart, incrementa/decremental temperature programming, computer linking via RS232 and a manual pause button.

*These notes are compiled by Brendan Horton from information provided by the manufacturers. For more details, fill in the reader service card bound inside the journal.*

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